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13. ABSTRACT (Maximum 200) Tumor suppressor protein p53 interacts directly with the DNA replication factor RPA and inhibits its ability to bind single-strand DNA. We defined the domain of p53 that bound to RPA and constructed p53 mutants that failed to bind RPA, but still functioned as transcriptional activators. We found that while these p53 mutants lost their ability to bind RPA, they still maintained the growth suppression function of p53. Growth suppression function of p53 is dependent on its transactivation activity, probably by inducing p21 and other cell cycle inhibitors. We have extended our study to the p21 protein, which is induced by p53 and interacts with both the cdk2 kinase and a DNA replication factor PCNA. We have demonstrated the importance of both cyclin/cdk-inhibitory domain and PCNA-inhibitory domain for the growth suppression function of p21 <i>in vivo</i> . We have also shown that p21 has to interact directly with both cyclin subunit and cdk2 subunit of the cyclin-cdk complex in order to inhibit the kinase activity and suppress cell growth <i>in vivo</i> . Furthermore, p21 can disrupt the interaction between PCNA and hFen1, an interaction important for the maturation of the newly replicated DNA.				
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FOREWORD

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TABLE OF CONTENTS

Front cover	1
Standard Form 298	2
Foreword	3
Table of contents	4
Introduction	5-6
Body	7-18
Conclusions	19-20
References	21-22
Bibliography	23

The following work is done under the supervision of Dr. Anindya Dutta, who is currently supported by a Career Development grant (DAMD17-94-J-4064). I am a postdoctoral fellow in Dr. Anindya Dutta's lab. Our independent grants only support our own salaries. There is some overlap in the work described below because it is being done in concert by postdoctoral fellow and advisor.

The first part of the work is focused on the interaction of tumor suppressor p53 and Replication Protein A (RPA), based on the earlier finding by Dr. Dutta that p53 can interact with RPA and inhibit its function. Our interests in the function of p53 in the development of breast cancer led us to investigate another important cell cycle regulatory protein p21, which is the major downstream regulatory protein of p53. p21 not only inhibits cyclin/cdk kinase activities, but also interacts directly with and inhibits an essential DNA replication factor, proliferating cell nuclear antigen (PCNA). Here we report our studies on p21 as the second part of this report.

INTRODUCTION

Tumor Suppressor p53 has been the major focus of the cancer research, because it is mutated in more than 50% of human cancers including breast cancer (1). There is significant evidence suggesting the importance of p53 in the cancer development: deletion of p53 gene in "knock-out" mice confers a very high risk of cancer; several viral oncogenes, including E6 gene of human papilloma virus and large T antigen of Simian virus 40, can specifically inactivate p53 by various mechanisms; cellular oncogene mdm2 can specifically bind to the N-terminal part of p53 and inactivate its transactivation activity. p53 has multiple biological functions: overexpression of p53 can arrest cell cycle at G1 to S transition; p53 is essential for DNA repair after mild DNA damage; p53 is also required for apoptosis following extensive DNA damage. The transforming mutants of p53 are defective in all these functions.

The mechanisms by which p53 carry out these biological functions are still unclear. p53 is a transcriptional activator, that has been shown to activate a number of cellular genes including cellular oncogene mdm2, DNA repair gene GADD45 and cell cycle regulatory p21 gene. p53 can also bind to TATA-box binding protein TBP, and suppress general transcription from promoters lacking p53 binding sites. Recently, more biochemical activities of p53 have been identified: p53 can bind to insertion-deletion mismatch lesions (2); p53 is also found to have 3' to 5' exonuclease activity (3). Our group (4) found that p53 could directly bind to DNA Replication Protein A (RPA) and inhibit its activity. This finding suggested a novel mechanism of p53 function, that is inhibiting DNA replication by directly interacting with RPA.

To test whether the ability to interact with RPA is important for the replication inhibitory property of p53, we mapped the interaction domains of RPA and p53. By direct mutagenesis, We obtained mutations of p53 that failed to bind RPA but still function as a transcription activator. These p53 mutants retain their transcriptional suppression and growth suppression functions. Therefore, the ability to bind RPA is not important for the growth suppression function of p53.

Our interests in the function of p53 in the development of breast cancer led us to investigate an important cell cycle regulatory protein p21. Transcription of p21 is induced by p53 upon DNA damage (5). "knock-out" experiment has shown that p21 is required for cell cycle arrest upon DNA damage (6). Overexpression of p21 can arrest cells at G1-S transition just like p53 (5, 7, 8). All these data suggest that p21 protein is a major effector of the growth suppression function of p53. Because of the importance of p21 as the major down-stream effector of p53, we have begun studying the mechanisms by which p21 arrests cell cycle. In the last two years of this project, we emphasized p21 and studied the mechanisms by which p21 inhibits cell growth.

The transition from G1 to S phase of the cell cycle is controlled by the activation of several cyclin/cdks. p21/CIP1 can inhibit all cyclin/cdk complexes. p21 has also been shown to interact directly with and inhibit an essential DNA replication factor, proliferating cell nuclear antigen (PCNA) (9, 10, 11, 12). Here, we report that the region of p21 involved in interacting with and inhibiting cyclin/cdk complexes can be separated from the region that interacting with

and inhibiting PCNA (12). We have further shown that both cyclin/cdk inhibitory activity and PCNA-inhibitory activity contribute to the growth suppression function of p21 (12, 27).

The cyclin/cdk inhibitory region of p21 can inhibit cell-cycle progression (12). This has interesting implications for the activities of other cdk inhibitors like p27 (up-regulated by TGF β , (13, 14)) and p57 (15, 16) which show homology with p21 only in the cyclin/cdk inhibitory region. In order to understand the mechanism by which p21 inhibits the cyclin/cdk complexes, we further narrowed down the cdk2 inhibitive domains. Here, we report that p21 can bind directly to both cyclins and cdk2. We further demonstrate that both cyclin-binding domain and cdk2-binding domain are important for the kinase inhibitory activity and growth suppression activity of p21 (29). That p27/p21 interacted with both cyclin and cdk2 was confirmed by the co-crystal structure of p27-cyclin A-cdk2 (17).

We have shown that p21 can inhibit cell growth by inhibiting PCNA (27). Because of the important function of PCNA in DNA replication and DNA repair, we searched for human gene products that interact with PCNA in a yeast two-hybrid system. Here, we have shown that human Fen1 associates with human PCNA *in vivo* and *in vitro*. p21 can disrupt the PCNA-Fen1 association *in vivo*. This suggests additional mechanisms by which p21 inhibits DNA replication (30). We also identified another DNA repair protein, human homolog of MutY, as a potential protein that interacts with PCNA. We are currently examining whether human MutY interacts with PCNA *in vivo* and whether p21 affects the function of MutY-PCNA complex.

BODY

Time Schedule:

Year 1:

1. Define the part of p53 involved in binding RPA and mutate it to obtain p53 mutants that do not bind RPA.
2. Analyze the effect of these mutations on the transcriptional property of p53.
3. Define the part of Rpa1 that binds p53.
4. Examine whether the p21-PCNA interaction contributes to growth suppression function of p21.

Year 2:

1. By mapping the interaction domains of RPA p70 and p53, several p53 mutants that abolish the binding to RPA p70 were constructed. We analyzed in detail the effect of these mutations on the transcriptional suppression and growth suppression activities of p53.
2. Examine whether the p21-PCNA interaction contributes to growth suppression function of p21.
3. Study the mechanism by which p21 inhibits cyclin-cdk2 complexes.

Year 3:

1. Examine whether the cyclin-binding motif of p21 can facilitate the formation of p21/cyclin/cdk complexes *in vivo*.
2. Examine whether p21 can disrupt associations of other cell cycle regulatory proteins with cyclin/cdk complexes by utilizing the cyclin-binding motif.
3. Study the mechanism by which p21 inhibits PCNA activities.

Materials and Methods

Yeast Two-hybrid Screen.

For the yeast two-hybrid screen, the human PCNA open-reading frame was cloned by PCR and inserted into the pAS2 vector to create a fusion protein with the GAL4 DNA-binding domain. pAS2-hPCNA was transformed into yeast strain Y190. For screening, a B cell cDNA library in the pACT vector was transformed into Y190 strain containing pAS2-hPCNA.

Expression Plasmids and Baculoviruses.

Plasmids utilized for expression of various proteins in bacteria were pETp21His, pETp27His, pETCdk2, pGST-RbC, pGST-cyclin D1,2,3 (Dr. Yue Xiong), pGST-cyclin E. A BamH I-Hind III fragment of cyclin E from pGST-cyclin E was cloned into the E. coli expression vector pRSET (Invitrogen) to generate pRSET-cyclin E (26-402). pGST-cyclin E (1-127) was generated by removing the Pst I-Hind III fragment from pGST-cyclin E. Similarly, pGST-cyclin E (1-216), (1-326) and (1-334) were generated by removing Nco I-Hind III, Nco I (2nd)-Hind III, Sac I-Hind III fragments, respectively.

Plasmids containing the mutant alleles of p21 were obtained from Dr. J. Smith. PCR with N- and C- terminal oligonucleotides was used to clone these mutant alleles of p21 into pGEX-5X-3. pGEX-p21Δ1-29 was made by restriction enzyme digestion of a plasmid expressing GST-p21 using a Pvu II site in the coding region of p21. pGEXp21Δ17-24 was made by PCR based strategy using appropriately designed oligonucleotides. Fragments containing p21

coding regions with deletions at either amino acids 17-24 or amino acids 53-58 also were cloned into pETHis for expression of His-tagged p21 derivatives in bacteria.

pGST-PCNA was constructed by ligating the full PCNA coding sequence into pGEX-5x-3 (Pharmacia) between BamHI and SalI sites. pGST-p21C2 expresses the C-terminal 39 amino acids of p21 fused to GST. pGST-Fen1C was constructed by ligating the sequence encoding the C-terminal amino acids of human Fen1 (residues 307-380, a BclI-SalI fragment) into the pGEX-5x-3 (BamHI and SalI). Plasmids were transformed into *E. coli* strain BL21, cell lysis and purification of GST-fusion proteins were done as described in (12).

The cDNA encoding the full length human Fen1 was a gift from Dr. J. Murray. pET-Fen1 was constructed by ligating the Fen1 full length cDNA (as a Nco I-Sal I fragment) into pET3a. pET-Fen1B was constructed by ligating the Nco I-BamH I fragment of Fen1 cDNA into pET3a, resulting in the deletion of C-terminal 17 amino acids from full length Fen1. All plasmids were transformed into *E. coli* strain BL21 for protein expression.

pCMVCdk2, pCMVCdk4, pRcCyclin A, pRcCyclin B, pRcCyclin E, pRcCyclin D1, D2, D3 were kindly provided by Dr. P. Hinds, and used for the expression of cyclins and Cdk's by *in vitro* transcription and translation.

Baculoviruses expressing Cdk2, GST-cyclin A, GST-cyclin B and GST-cyclin E were obtained from Dr. Helen Piwnicka-Worms. Baculoviruses expressing cyclin D1, 2, 3 were gifts from Dr. C. J. Sherr. Baculoviruses expressing GST-Cdk4 was provided by Dr. J. W. Harper.

Mammalian cell expression constructs containing full length p21 and p21N, p21(Δ 17-24) and p21N (Δ 17-24) were cloned into pcDNA3 (Invitrogen). The fragments containing full length Fen1 and Fen1B were generated by PCR using Pfu polymerase (Stratagene) and cloned into pA3M vector to generate pA3M-Fen1 and pA3M-Fen1B for expression of myc epitope tagged proteins in human cells.

Protein expression and purification.

Bacterial expression of proteins was performed in *E. coli* strain BL21. Protein induction, cell lysis and affinity-purification with glutathione-agarose beads (Sigma) were done as described (12). Bacterially expressed Fen1 was purified by chromatography on a DEAE-sepharose column in buffer A (20 mM Hepes pH8.0, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, 10% glycerol) containing 0.3 M KCl (Flow-through) followed by binding to S-sepharose at 0.1 M KCl and elution with 0.3 M KCl. This crude preparation of Fen1 protein was dialyzed against buffer A, and loaded on 5 ml Mono-Q FPLC column (Pharmacia). Proteins were eluted with 50 ml of buffer A with a salt gradient of 0-1 M KCl. Fen1 peak was followed by SDS-PAGE and Coomassie staining. For Fen1B purification, the flow through from the DEAE-column was loaded on Q-sepharose column (Pharmacia) equilibrated with buffer A containing 0.3 M KCl. The flow through from the Q-sepharose was loaded on 5 ml Hydroxylapatite column (0.1M KCl) and eluted with 15 ml buffer A/0.1M KCl /80 mM Sodium phosphate. Eluted Fen1B was then dialyzed overnight against buffer A (pH6.0) containing 25 mM KCl, loaded on 5 ml S sepharose column and eluted with buffer A (pH6.0) containing 100 mM KCl. Purified Fen1 or Fen1B were dialyzed against buffer A7.4 (20 mM Tris-HCl pH7.4, 1 mM EDTA, 0.01% NP40, 10% glycerol, 25 mM NaCl, 1 mM DTT, 0.1 mM PMSF).

Hi-5 cells were infected with recombinant baculoviruses containing various cyclins and cdk's as described (12). Active cyclin/Cdk complexes were affinity-purified with glutathione-agarose beads (Sigma).

Protein expression by *in vitro* transcription and translation were performed using TnT coupled Rabbit Reticulocyte Lysate system (Promega).

Peptides (PS100-PS103) were synthesized by Research Genetics Inc. PS100: ACRRLFGPVDSE; PS101: ACRRLKKPVDSE; PS102: FYHSKRRLIFSK; PS103: FYHSKRDDIFSK. A 41 amino acid p21C2 peptide (consisting of the 39 C-terminal amino acids of p21 and two lysine residues at the carboxy-terminal end required for chemical synthesis) was synthesized at the Harvard Medical School Biopolymer Laboratory.

p21C2: QAEGSPGGPGDSQGRKRRQTSMTDFYHSKRRLIFSKRKPKK
BWH262: WNSGFESYGSSSYGGAGGYTQAPGGFGAPAPSQAEKKSRAR (control peptide from N terminus of human RPA p34).

Gel Filtration and Glycerol gradient.

Proteins were incubated on ice for 15 minutes in A7.4 buffer before loading on 25 ml Superose 12 gel filtration column (Pharmacia). Proteins were eluted from the column at flow rate 0.4 ml per minute, and 0.5 ml fractions were collected.

5 ml 10%-40% glycerol gradients were made with the gradient maker. 50-100 μ l of proteins were carefully added on the top of the gradient. Gradients were centrifuged at 55,000 rpm using SW55 rotor for 12-24 hours in L8-M (Beckman). Fractions of 200 μ l were collected starting from the top of the gradients.

Pull down assay, immunoprecipitation and immunoblotting.

Pull down assays were performed as described (12). Basically, 100-300 ng of GST fusion protein and 5-10 μ l of bacterial or reticulocyte lysate in 200 μ l of buffer A7.4 (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.01% NP40, 10% glycerol, 25 mM NaCl) were incubated one hour at 4 degrees on a rotating wheel. Proteins associated with GST fusion proteins were pulled down with glutathione agarose beads. After washing the beads 4 times in binding buffer, bound proteins were eluted by boiling in Laemmli's SDS-PAGE sample buffer for 10 minutes, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and detected either by autoradiography (for radiolabeled proteins expressed by in vitro transcription and translation reactions using rabbit reticulocyte lysate) or by western blotting using appropriate antibodies. In each experiment, care was taken to equalize the amount of input proteins and GST protein was included as a negative control. For peptide competition, the indicated peptides were included in the reactions at a concentration of 75 μ M.

The antibodies used in this study were monoclonal antibodies to cyclin E (HE12, HE172) and polyclonal antibodies to p27 (J. Massague), cdk2 (Santa Cruz), cdk4 (H. Chou), cyclin A (J. Pines), and cyclin D1 (L. Zuckerman). The monoclonal antibodies (CP2, CP36, CP68) to p21 were generated against full length recombinant human p21. CP2, CP36, CP68 recognize the amino acids 1-17, 17-24 and 130-150 of p21, respectively. This was determined by immunoblotting a panel of bacterially produced deletion derivatives of p21. Anti-Fen1 polyclonal rabbit antibody was raised against purified bacterial expressed Fen1 (Cocalico) and affinity purified. PCNA was detected by Western blot using a monoclonal antibody (PC10; Santa Cruz Biotechnology). The Myc-tagged Fen1 or Fen1B were transfected into 293T cells by standard calcium phosphate coprecipitation method. Thirty-six hours after transfection, cells were lysed in NP-40 lysis buffer and Myc-tagged proteins were immunoprecipitated using antibody (9E10) against Myc-epitope.

Kinase assay.

Kinase assays were performed for 15 min at 30°C using 1 ng of insect cell-expressed cyclin/Cdk complexes and 3 μ g of purified GST-RbC (C-terminal portion of Retinoblastoma protein Rb) in 25 ml of kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM DTT and 50 μ M ATP containing 5 μ Ci [g-³²P] ATP) with other indicated components. Kinase reactions were stopped by the addition of 2X Laemmli sample buffer and then boiled for 10 minutes. The products were analyzed by SDS-PAGE. Phosphorylation of the substrate was quantitated both by liquid scintillation counting of bands excised from gels and by phosphorimager analysis. Ki_{app} is the concentration of inhibitor at which kinase activity is inhibited by 50%.

For immunoprecipitation-kinase assays shown in Fig. 2, 10 ng of GST-cyclin E/cdk2 was incubated with 1 µg of bacterially produced p21 derivatives (or peptides) at 4°C for 30 min in 200 µl of buffer A7.4. p21-associated proteins were immunoprecipitated with monoclonal antibody CP68 and kinase reactions were performed with GST-RbC as the substrate.

Growth suppression assay.

The stable transfection assay for measuring growth suppression by p21 and its alleles was performed as described (12).

RESULTS

Studies on the interaction of p53 and RPA:

Earlier study in from our lab showed that tumor suppressor p53 could interact with Replication Protein A (RPA) and inhibit its function as a single strand DNA binding protein. We propose that p53 inhibits entry into S phase by its interaction with RPA. To test this hypothesis, we defined the domain of p53 that binds RPA. Deletion mutagenesis showed that N-terminal domain (amino acids 2-71) of p53 was necessary and sufficient for binding of p53 to RPA in crude cell extract. Point-mutations in amino acids 53-54 (W53S-F54S) disrupted the interaction. Mutations in amino acids 48-49 (D48H-D49H) also decreased RPA binding. The mutations which changed amino acids 22-23 of p53 (L22Q-W23S) affected its ability to activate transcription, but did not affect its ability to bind RPA. Transient transfection assay was done to test the transcription activation and suppression properties of these p53 mutants. Only the L22Q-W23S mutation of p53 significantly affected transcription activation and growth suppression properties of p53. D48H-D49H and W53S-F54S mutant forms of p53 retained 50-100% of transcriptional activity compared to wild-type p53. Therefore we separated the transactivation/suppression and RPA binding functions of p53 with appropriate point mutations.

To examine the growth suppression activity of p53, plasmids expressing wild type or mutant p53 were transfected into SaOs2 and H1299 cells (deficient in endogenous p53) and G418 resistant colonies were selected. Plasmids expressing wild type p53 established very few G418 resistant colonies compared to the vector, due to growth suppression by p53. Two mutants of p53 (D48H-D49H and W53S-F54S) inhibit cell growth as well as wild type p53, indicating that RPA-p53 interaction is not required for the growth suppression activity of p53 (25).

The p53 protein with the mutation of amino acids L22Q-W23S, which had wild type RPA binding activity but reduced transcriptional activation and repression activities, showed diminished growth suppression in both SaOs2 and H1299 cells. The L14Q-F19S and D61H-E62K mutants, which retained most of the transcriptional activation and repression functions, also retained most of the growth suppression activity of wild-type. These results imply the transactivation and/or repression properties of p53 are important for its growth suppression function.

Studies on p21, a downstream effector of p53:

p21 (WAF1, CIP1 or sdi1) is a protein induced by the tumor suppressor protein p53, which interacts with and inhibits two different targets essential for cell-cycle progression. One of these is the cyclin/cdk kinases and the other is the essential DNA replication factor PCNA. Because of our primary interest in the role of p53 in the tumor development, we expanded our studies on p21, the major downstream regulator of p53.

Separate domains of p21 involved in the inhibition of cyclin/cdk kinases and PCNA.

When the N or C terminal halves of p21 (p21N or p21C) were expressed separately as GST fusion proteins, p21N (but not p21C) associated with cyclin/cdk2 and inhibited the kinase activities of cyclin A/cdk2 or cyclin E/cdk2. p21C (but not p21N) associated with PCNA and inhibited the SV40 based *in vitro* DNA replication. Thus separate domains of p21 are involved in the inhibition of cdk kinases and PCNA (12).

Inhibition of *Xenopus* DNA replication by different domains of p21.

p21 inhibits DNA replication of sperm in interphase extracts derived from *Xenopus* eggs. p21N inhibits DNA replication in the *Xenopus* extract at the same concentrations as p21, which are similar to those of cyclin E/cdk2 (100 nM). Thus, in *Xenopus* extracts it appears that cyclin/cdk2 rather than PCNA is limiting and is inhibited by p21. High concentrations of p21C,

approximating that of PCNA in the extract (10 μ M), inhibited *Xenopus* DNA replication. Therefore, PCNA is also required for double-stranded DNA replication and can be inhibited by p21, but is not the limiting factor inhibited by the addition of p21. In contrast to double-stranded DNA replication, DNA synthesis on single-stranded DNA was not inhibited by the cdk inhibitory p21N domain, but was inhibited by p21C at concentrations approaching that of PCNA. Therefore, active cdk kinase is required specifically for DNA synthesis on double-stranded DNA, while PCNA is required for DNA synthesis on both types of substrates (12).

Growth suppression assay shows that p21N inhibits cell growth.

Using these separated domains, we have determined that p21 inhibits different biological systems through different targets. The question arises as to which of the two targets, cdks or PCNA, mediates the growth suppression function of p21. The N terminal cyclin/cdk2 inhibitory domain (p21N) can suppress cell growth as well as the wild type p21, while the C terminal PCNA inhibitory domain (p21C) did not. These results suggest that cyclin/cdks are the primary targets of p21 in transformed cells (12).

Cyclins associate stably with p21 or p27 independent of the catalytic cdk2 subunit.

As we showed above, the primary growth suppression property of p21 is due to its ability to inhibit cyclin/cdk complexes. p21 can bind directly to the cdk2 subunit of the cyclin/cdk complex, we tested whether cyclin subunit can also interact directly with p21. Using bacterially expressed p21 or p27 and various cyclins, we demonstrated that p21 or p27 could associate with cyclins in the absence of any cdk catalytic subunit, and these interactions depended on a highly conserved sequence, ACRRLF^{GP}, among the cdk inhibitors p21, p27 and p57 (29).

The Cyclin-binding motifs of p21 are important for interacting with and inhibiting cyclin/cdk kinases.

Using bacterially expressed p21 or deletion derivatives and various sources of cyclin/cdk complexes, we have shown the cyclin-binding motifs of p21 are important for its association with various cyclin/cdk complexes (29). We have also shown that the cyclin-binding motifs of p21 are required for the inhibition of various cyclin/cdk kinases *in vitro* (29).

The Cyclin-binding motifs of p21 are required for cell growth suppression.

To determine whether the cyclin-binding motifs of p21 were important for the biological activity of p21 *in vivo*, we tested cell growth suppression by various deletion derivatives of p21. Plasmids expressing p21 (Cy1⁺, K⁺, Cy2⁺), p21 Δ 17-24 (Cy1⁻, K⁺, Cy2⁺), p21N (Cy1⁺, K⁺, Cy2⁻) and p21N Δ 17-24 (Cy1⁻, K⁺, Cy2⁻) were stably transfected into SaOs2 cells (p53 null, Rb null) and H1299 cells (p53 null, intact Rb) and colony formation measured. In agreement with the *in vitro* results, only p21 with neither Cy1 nor Cy2 sites (p21N Δ 17-24) significantly lost growth suppression (29).

Cyclin-binding motif of p21 is required for the formation of p21/cyclin/cdk2 complexes *in vivo*.

We expect the cyclin-binding motifs of p21 to be important for the formation of p21/cyclin/cdk complexes *in vivo* at physiological concentration of p21. CP36 is a monoclonal antibody that fails to recognize p21 Δ 17-24, indicating that its epitope overlaps with the cyclin-binding motif 1 (Cy1) of p21 (Fig. 1A). Pre-incubation of p21 with this antibody selectively disrupts the association of recombinant GST-cyclin E/cdk2 with p21 in a pull-down assay (Fig. 1A). However, the association of p21 with GST-cyclin A/cdk2 is not affected by the antibody.

CP68, an antibody recognizing the C terminus of p21, and CP2, an antibody recognizing the N terminal 17 amino acids of p21, did not block the association of p21 with cyclin E/cdk2 or cyclin A/cdk2 (Fig. 1A and data not shown). As cyclin A/cdk2 and cyclin E/cdk2 have the same cdk2 subunit, the differential effect of CP36 on the association of p21 with either cyclin A/cdk2 or cyclin E/cdk2 confirms that the antibody disrupts an interaction between the cyclins and p21. CP36 antibody allowed us to test whether the Cy1 site is important for p21/cyclin/cdk complex formation in cells. CP36, CP68 and CP2 antibodies were used to immunoprecipitate p21 from cell extracts and co-precipitated cyclins and cdks detected by immunoblotting (Fig. 1B). CP36 specifically failed to co-immunoprecipitate cyclins E, D1 and cdk4 protein with p21 from cell extracts, confirming that the Cy1 region of p21 is utilized *in vivo* for interaction with these proteins (29). Cyclin A and cdk2 were present in the CP36 immunoprecipitates in agreement with *in vitro* association results shown in Fig. 1A.

Association of p21 with cyclin/cdk through cyclin-binding motif allows partially active cyclin/cdk kinase to be associated with p21.

Although p21 was identified as an inhibitor of cyclin/cdk kinases, active cyclin/cdk is found complexed with the cdk inhibitor p21 in cell extracts (21). Our results suggest that complete kinase inhibition requires association of the cyclin/cdk complex with at least one cyclin-binding site and the cdk2-binding site of p21. However, stable association can occur with p21 containing only cyclin-binding sites. Therefore, it is conceivable that partially active cyclin/cdk kinase may be complexed with p21 through the cyclin-binding sites. To test this hypothesis, we mixed wild type p21, p21 Δ 17-24 (Δ Cy1) and p21 Δ 53-58 (Δ K) with GST-cyclin E/cdk2 and immunoprecipitated the p21-cyclin/cdk complexes with the anti-p21 antibody CP68. Equal amounts of GST-cyclin E were detected in the three immunoprecipitates by immunoblotting (Fig. 2, left bottom). However, the only immunoprecipitate containing active kinase was the one with p21 Δ 53-58 (Δ K) (Fig. 2, left top). The association was selectively disrupted by the PS100 peptide (Fig. 2, right). Therefore, partially active cyclin E/cdk2 kinase stably associates with p21 through the cyclin-binding motifs.

Addition of wild-type p21(Cy1⁺, K⁺, Cy2⁺) to the immunoprecipitate of p21 Δ 53-58-cyclin E/cdk2 inhibited, but p21 Δ 17-24 (Cy1⁻, K⁺, Cy2⁺) failed to inhibit, the residual kinase activity (data not shown). Therefore the cdk2-binding site cannot be provided in trans to cyclin/cdk2 already complexed with p21 through the Cy sites. We have not observed any additive inhibition when GST-p21 Δ 17-24 (cdk2-binding site alone) and PS100 peptide (cyclin-binding motif 1 site alone) were added simultaneously to a kinase reaction (data not shown). Thus, there is no evidence yet that the cyclin-binding and cdk2-binding activities can be provided in trans by two different p21 molecules (29).

C-terminal part of p21 can prevent quiescent cells entry into S phase.

To determine whether C-terminal of p21 can interact with and inhibit PCNA *in vivo*, we analyzed whether entry into S phase could be inhibited by various p21 derivatives. Quiescent diploid fibroblasts were stimulated by serum and entry into S phase followed after micro-injection of GST-fusion proteins. GST-p21, GST-p21N and GST-p21C inhibited uptake of Bromodeoxyuridine significantly compared to a negative control peptide CSH119, GST alone, or GST fused to a cell-cycle regulatory protein cdc25C. Thus, GST-p21C inhibits growth of cells almost as well as GST-p21N when provided in high enough concentrations. These results not only confirm earlier reports that p21N, which binds and inhibits cdk kinases but not PCNA, inhibits cell growth, but also suggest that p21C, which binds and inhibits PCNA, can also inhibit cell growth if provided at high concentration (27).

Human Fen1 associates with PCNA through its C-terminal tails.

As PCNA is important for DNA replication and DNA repair, a yeast two-hybrid screen was carried out to search for human gene products that interact with human PCNA. Human Fen1 associates with PCNA in the yeast two-hybrid assay. We have shown that three molecules of Fen1 form a stable complex with the PCNA trimer *in vitro* and this interaction is dependent on the C-terminal basic tail of Fen1 (30). We have also shown that the Fen1 and PCNA associate with each other *in vivo* under physiologically conditions, and this *in vivo* interaction is also dependent on the C-terminal tail of Fen1 (30).

p21 displaces Fen1 from the Fen1-PCNA complex.

Since p21 also interacts with PCNA using a basic region of p21 similar to that used by Fen1, we tested whether the two molecules compete for the same binding site on PCNA. p21 displaced PCNA from the GST-Fen1C molecule (Fig. 3A). Further, a synthetic peptide that contains the C terminal 39 amino acids of p21 and that interacts with and inhibits PCNA, p21C2 (27), also disrupted the Fen1C-PCNA complex.

The mutually exclusive complex formation between PCNA-Fen1 and PCNA-p21 were further demonstrated by gel filtration assay (Figure 3B). GST-p21C2 (31 kD) is a fusion of the last 39 amino acids of p21 with GST and associates well with PCNA (27). When GST-p21C2, Fen1 (45 kD) and PCNA (120 kD) were mixed and analyzed on a gel filtration column, all three proteins eluted in the same fractions of about 150 kD (Figure 3B). However, when the GST-p21C2 was precipitated from these fractions with glutathione agarose beads, only PCNA was co-precipitated (Figure 3C), suggesting that the three proteins were present in two separate complexes each of about 150 kD: GST-p21C2-PCNA and Fen1-PCNA. A tripartite Fen1-p21-PCNA complex was never observed.

To demonstrate that p21 can disrupt Fen1-PCNA interaction *in vivo*, Myc-tagged Fen1 was expressed alone or with p21 in the cells. While Myc-tagged Fen1 associated with PCNA *in vivo*, co-expression of p21 in these cells disrupted Fen1-PCNA interaction (Fig. 3D).

FIGURE LEGENDS

Figure 1. A) Epitope mapping: 100 ng bacterially expressed p21 or p21 Δ 17-24 were immunoblotted with the CP36 and CP68 antibodies. CP68 recognizes the 130-150, and CP2 recognize 1-17 region of p21 (data not shown). In vitro association: Bacterially produced p21 (200 ng) was pre-incubated on ice with 50 μ l of hybridoma culture supernatants containing indicated antibodies (none, CP36 or CP68) for 30 min. and then incubated with indicated GST-cyclin/cdk2 complexes (1 μ g) bound to glutathione agarose. p21 bound to the beads was visualized by immunoblotting with anti-p21 monoclonal antibody CP36. B) Immunoprecipitation from cell extracts: Equal amounts of WI38 cell extracts were immunoprecipitated with CP36 or CP68 antibodies (covalently coupled to beads) and the precipitates immunoblotted to detect the indicated proteins.

Figure 2. Association with p21 through only the cyclin subunit allows partially active kinase to be complexed with p21. Associated kinase activity: Wild type, Δ 17-24 or Δ 53-58 p21 (mutated in the cdk2-binding site) was mixed with GST-cyclin E/cdk2, immunoprecipitated with CP68 antibody and the activity of the kinase in the precipitate tested on GST-RbC. For the panel on the right, the Δ 53-58 form of p21 was mixed with cyclin E-cdk2 kinase in the presence of no peptide (lane 4), the wild type PS100 peptide (lanes 5-7) or the mutated PS101 peptide (lanes 8-10) added at the amounts indicated on top (μ g/ 200 μ l). Associated proteins: The presence of equivalent amounts of GST cyclin E (lanes 1-4 and 8-10) and absence of GST-cyclin E (lanes 5-7) in the immunoprecipitates was verified by immunoblotting with anti-cyclin E antibody HE12. Immunoblot with anti-cdk2 antibody showed that cdk2 levels paralleled that of cyclin E (data not shown). The bands below GST cyclin E are immunoglobulin heavy chain present in the immunoprecipitates.

Figure 3. p21 disrupts Fen1-PCNA interaction. A. Bacterial lysate containing human PCNA was incubated with the GST-Fen1C or with GST immobilized on the agarose beads in a pull-down assay. PCNA bound to the beads were detected by immunoblot with anti-PCNA antibody. Lane 1, 10% input lysate; lanes 2-10, GST-Fen1C beads incubated with bacterial lysate containing PCNA with no additional protein (lane 2), with additional Hisp21 (lane 3-5: 0.5, 1 and 2 μ g), p21C2 peptide (lane 6-8: 0.5, 1 and 2 μ g), Hisp27 (lane 9: 2 μ g) or control peptide CSH190 (lane 10: 1.5 μ g); lane 11: GST beads incubated with PCNA containing lysate. B. 25 mg PCNA (36 kD), 25 mg Fen1 (45 kD) and 50 mg GST-p21C2 (31 kD) were mixed and analyzed on a Superose 12 gel-filtration column. C. 200 μ l of indicated fractions from (B) were incubated with glutathione agarose beads, bound proteins (GST-p21C2 and associated proteins) were separated by SDS-PAGE and visualized by Coomassie staining. D. Cells were transfected with Myc-Fen1 alone, Myc-Fen1 and p21 or vector alone and collected after 36 hours. Cell-lysates were immunoblotted with anti-Myc and anti-p21 antibodies to demonstrate that equal levels of Myc-Fen1 are expressed in the first two lanes (Left). Lysates were immunoprecipitated with anti-myc antibody 9E10 and precipitates immunoblotted with anti-PCNA antibody to demonstrate that coexpression of p21 decreases Myc-Fen1-PCNA association (Right).

Figure 1

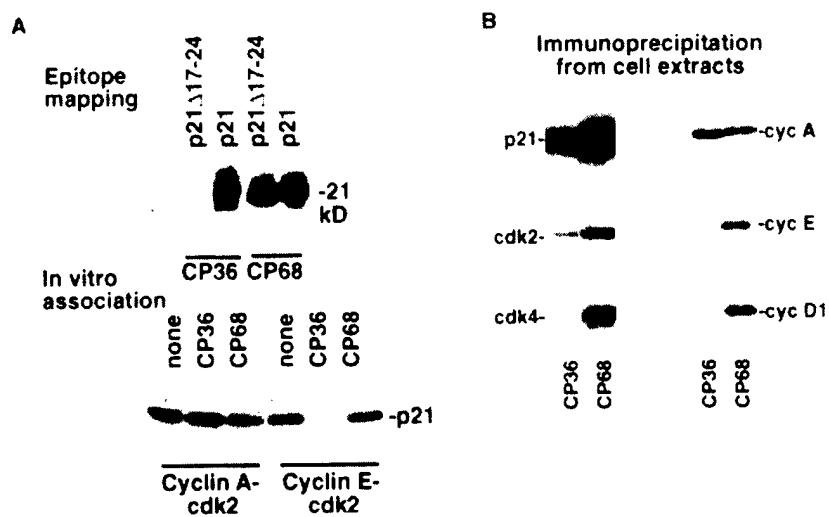


Figure 2

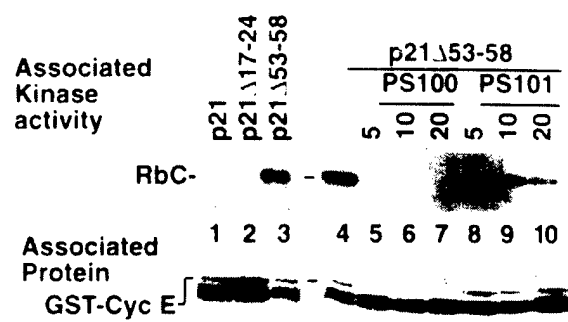
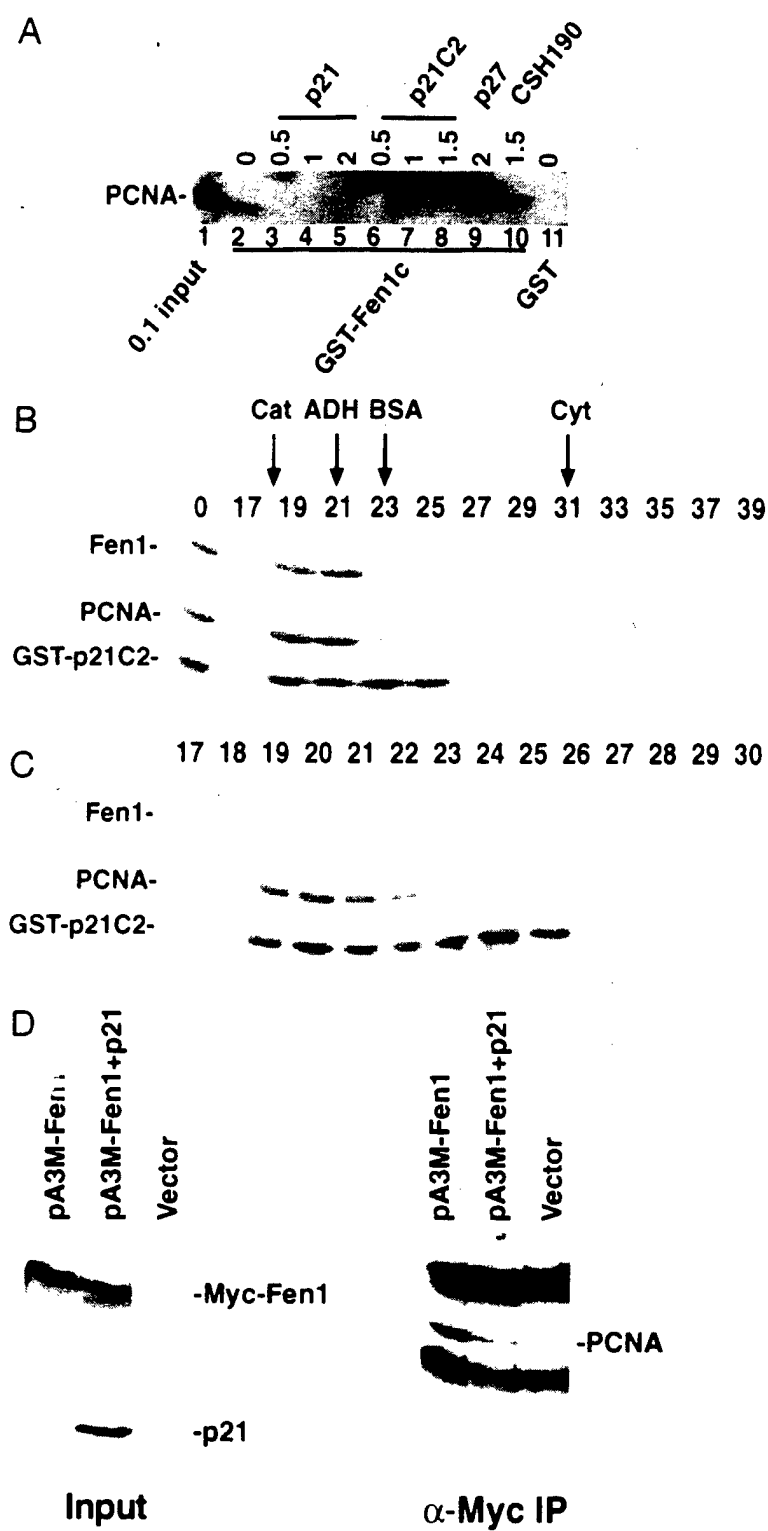


Figure 3



CONCLUSIONS

In this study, we defined the domain of p53 that bound to RPA and constructed p53 mutants that failed to bind RPA, but still functioned as transcriptional activators. We examined whether these p53 mutants affected other functions of p53, including transcriptional suppression and growth suppression activities. We found that while these p53 mutants lost their ability to bind RPA, they could still function as transcriptional suppressor on the promoters lacking the p53 binding site and suppress cell growth in two tumor cell lines we tested. These results suggest that RPA-p53 interaction is not required for the growth suppression activity of p53. Instead, transcriptional activation and/or repression activity of p53 is responsible for the growth suppression activity of p53. These results were published (25). p53 has other functions relevant to the cancer development and progression. p53 is required to induce apoptosis in response to irradiation or chemotherapy, to produce a pause in DNA replication after a sub-lethal dose of radiation so as to give the cell time to repair its DNA, and to prevent gene amplification. p53 can induce apoptosis independent of its transcriptional activation activity (26). p53 can also bind to insertion-deletion mismatch lesions (2) and function as an exonuclease (3). p53 may recruit RPA to these sites of DNA repair by p53-RPA interaction. Future experiments will be directed to test whether RPA-p53 interaction is important for these functions of p53.

The growth suppression activity of p53 is dependent on its ability to activate transcription. More and more evidence suggest that p21 is the major downstream effector of p53 in cell cycle control. p21 can be transcriptional up-regulated by p53 upon DNA damage. p21 can also suppress cell growth when overexpressed *in vivo*. In order to understand how p53, through regulating p21 level, controls the cell cycle upon DNA damage, we began to study how p21 directly inhibits the cell cycle machinery. Because p21 can interact with and inhibit both cyclin/cdk and PCNA, we first tested whether these two activities of p21 can be separated and whether both activities are required for the biological activity of p21. By using deletion mutations of p21, we separated domains of p21 responsible for interacting with and inhibiting cyclin-cdk and PCNA. We found that cdk kinases were the primary target for inhibition of double-stranded DNA replication in *Xenopus* extracts by p21 and for growth suppression in transformed cells while PCNA was the limiting target in the SV40 replication reaction (12).

In order to understand the mechanisms by which p21 inhibits the cyclin/cdk kinases, we further dissected the cyclin/cdk inhibitory domain. We report that p21 interacts directly with both cyclin and cdk subunits of the cyclin-cdk complexes. We have also demonstrated that p21 uses a highly conserved motif (we named it as cyclin-binding motif) to interact with cyclins (29). These results agree with the crystal structure of p27-cyclin A-cdk2 complex (17). The cyclin-binding motif is not only important for the kinase inhibitory activities of p21, but also important for the biological activities of p21. Cyclin-binding motifs are highly conserved among p21, p27, p57 family. Sequences similar to the cyclin binding motif are also found in other cell cycle regulatory proteins, such as p107 (22) and E2F1 (28). Based on our studies of p21 and crystal structure of p27-cyclinA-cdk2 complex, we suggest that the conserved cyclin binding motifs in p57, p107 and E2F1 interact specifically with cyclins. In some cases (p107, E2F1) the Cy motif may be an accessory site to target the substrate to a cyclin/cdk kinase. By using an antibody specifically recognizing the Cy1 site of p21, we have shown that the cyclin-binding site is important for facilitating the formation of p21/cyclin/cdk complexes *in vivo* (29). Recently, we found that CDC25A associated with cyclin E/cdk2 through a cyclin-binding motif and p21 can disrupt the association of CDC25A with cyclin E/cdk2 complexes by utilizing the cyclin-binding motif.

We also examined the importance of p21-PCNA interaction *in vivo* (27). By employing microinjection technique, we showed that PCNA-interacting domain or peptide can inhibit cell growth *in vivo* (27). In a yeast two-hybrid screening search for proteins interacting with human PCNA, we have found that human Fen1 associates with PCNA. Human Fen1 (also called MF1, Maturation Factor 1) has both exonuclease and endonuclease activities and is required for the maturation of Okazaki fragments during DNA replication. Overexpression p21 can disrupt

the interaction between hFen1 and PCNA, suggesting a mechanism by which p21 inhibits DNA replication. Recently, the co-crystal structure of p21-PCNA (32) suggest the association of p21 with PCNA does not alter the ring-shape structure of PCNA. The ability of p21 to inhibit the activity of PCNA is speculated to be due to its ability to prevent the associations of other proteins with PCNA (32). Fen1-PCNA association is a good example of these associations that are disrupted by p21. PCNA has also been shown to associate with mismatch repair proteins MLH1 and MSH2 (31). Recently, we identified another DNA repair protein, human homolog of MutY, that interacted with PCNA. We are currently investigating the biological relevance of this interaction and examining the role of p21 in regulating the associations of PCNA with various DNA replication and DNA repair proteins.

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Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA

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The protein p21 (WAF1, CIP1 or sd11), induced by the tumour-suppressor protein p53, interacts with and inhibits two different targets essential for cell-cycle progression¹⁻⁸. One of these is the cyclin-Cdk family of kinases and the other is the essential DNA replication factor, proliferating-cell nuclear antigen (PCNA). We report here that separate domains of p21 are responsible for interacting with and inhibiting the two targets. An amino-terminal domain inhibits cyclin-Cdk kinases and a carboxy-terminal domain inhibits PCNA. Using these separated domains, we have determined that p21 inhibits different biological systems through different targets. The PCNA-binding domain is sufficient for inhibition of DNA replication based on simian virus 40, whereas the Cdk2-binding domain is sufficient for inhibition of DNA replication based on *Xenopus* egg extract and for growth suppression in transformed human cells.

We have successfully separated the domains of p21 responsible for inhibiting Cdk2 and PCNA (Fig. 1). When the N- or C-terminal halves of p21 (p21N or p21C) were expressed separately

as GST fusion proteins, p21N alone physically associated with bacterially expressed Cdk2 kinase polypeptide in a standard 'pull-down' assay. Further, p21 and p21N, but not p21C, inhibited the kinase activity of cyclin A-Cdk2.

In contrast, the ability to interact with PCNA is the function of the C-terminal half of p21 (p21C). In a pull-down assay, bacterially expressed PCNA was quantitatively bound by p21 and p21C (Fig. 2). As reported, GST-p21 inhibited the simian virus 40 (SV40)-based *in vitro* DNA replication reaction (Fig. 2), and this inhibition was reversed by the addition of PCNA or a fraction of cell extract containing PCNA (refs 7, 8 and data not shown). The ability to inhibit the SV40-based DNA replication reaction required the PCNA-binding domain present

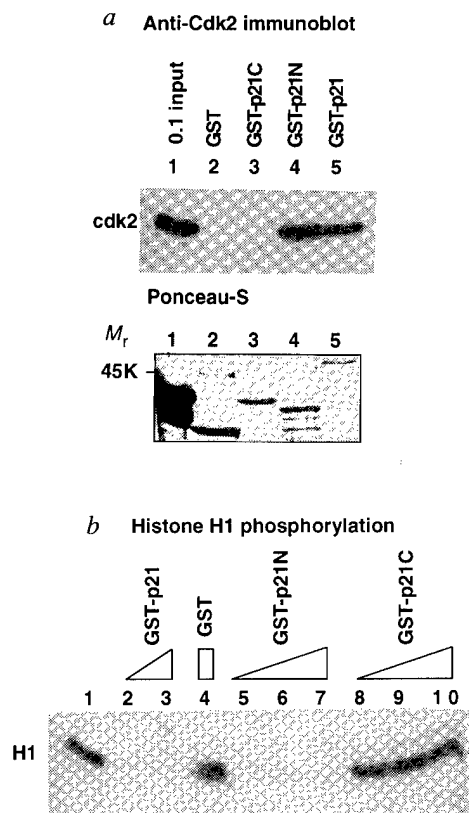


FIG. 1 The N-terminal half of p21 binds to and inhibits Cdk2 kinase. *a*, Immunoblot probed with anti-Cdk2 antibody shows which GST fusion proteins bind Cdk2. Ponceau S stain of the same blot demonstrates the relative amounts of the various GST fusion proteins present in each lane. The GST lane also contains M_r markers, and the 45K marker is indicated. The intense band in the 0.1 input lane is the carrier protein casein. *b*, Autoradiogram of proteins showing the phosphorylation of histone H1 by cyclin A-Cdk2 kinase with the following additions. Lane 1, no addition; 2, 3, 20 ng and 100 ng GST-p21; 4, 100 ng GST; 5-7, 20 ng, 100 ng, 200 ng GST-p21N; 8-10, 20 ng, 100 ng, 200 ng GST-p21C.

METHODS. Plasmids expressing GST fusion proteins in *Escherichia coli* were made by PCR of p21 and its deletion derivatives as *Bam*HI-SalI fragments, and cloning into the pGEX-5X-3 (Pharmacia). GST-p21 contains the whole coding region of human p21 gene (amino acids 1-164), GST-p21N contains amino acids 1-90, and GST-p21C contains amino acids 87-164. All p21 coding regions were preceded by a methionine. The expression and binding of GST fusion proteins to glutathione-agarose beads have been described before¹⁶. Cdk2 protein was expressed from *E. coli* using pET-Cdk2. The pull-down assay was done as previously described¹⁶, except that washing was done with buffer A. Histone H1 kinase assays were for 20 min at 30 °C using 10 ng of insect cell-expressed GST-cyclin A-Cdk2 complex and 8 µg of histone H1 in 20 µl kinase buffer¹¹. The phosphorylation of histone H1 was visualized by electrophoresis and autoradiography.

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in p21C (Fig. 2). Although the concentration of p21N added was sufficient to inhibit completely the endogenous histone H1 kinase activity in the extracts (data not shown), SV40 DNA replication was not inhibited.

p21 also inhibits DNA replication of sperm in interphase extracts derived from *Xenopus* eggs (P.K.J., S. Chevalier, M. Phillippe and M.W.K., manuscript submitted; also ref. 9). p21N inhibits DNA replication in the *Xenopus* extract at the same concentrations as p21 (Fig. 3a), which are similar to those of

cyclin E-Cdk2 (100 nM). Thus, in *Xenopus* extracts it appears that cyclin-Cdk2 rather than PCNA is limiting and is inhibited by p21. High concentrations of p21C, approximating that of PCNA in the extract (10 μ M), inhibited *Xenopus* DNA replication. Therefore, PCNA is required for double-stranded DNA replication and can be inhibited by p21, but is not the limiting factor inhibited by the addition of p21. In contrast to double-stranded DNA replication, DNA synthesis on single-stranded DNA was not inhibited by the Cdk inhibitory p21N domain but was inhibited by p21C at concentrations approaching that of PCNA (Fig. 3b). Therefore, active Cdk kinase is required specifically for DNA synthesis on double-stranded DNA, whereas PCNA is required for DNA synthesis on both types of substrates.

The question arises as to which of the two targets, Cdks or PCNA, mediates the growth suppression function of p21 in p53-null transformed cells in culture (Fig. 4a). As reported by others,

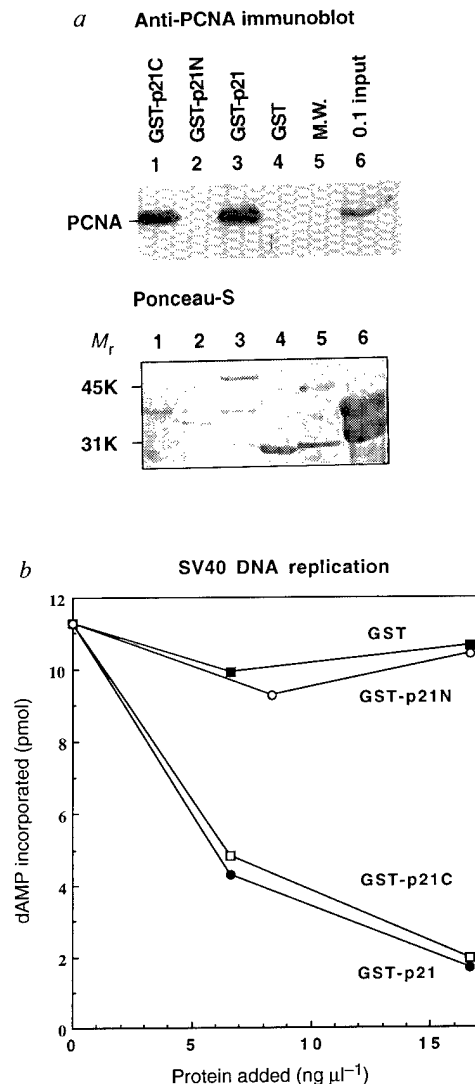


FIG. 2 The C-terminal half of p21 binds to PCNA and inhibits SV40-based DNA replication reaction. **a**, Immunoblot probed with anti-PCNA antibody (Oncogene Science) shows which GST fusion proteins bind PCNA. Ponceau S stain of the same blot demonstrates the relative amounts of the various GST fusion proteins present in each lane. **b**, Titration of various fusion proteins into SV40 DNA replication reactions containing SV40 T-antigen and a crude cell extract from human 293 cells. DNA synthesis is expressed as pmol of dAMP incorporated in a 50 μ l reaction in 1 h.

METHODS. PCNA protein was expressed from *E. coli* using pET-PCNA. Pull-down assay was exactly as described in Fig. 1. SV40 DNA replication was as previously described²¹. pSV011 (180 ng) was replicated in a 30 μ l reaction containing 100 ng SV40 T-antigen (obtained from baculovirus expression system and already phosphorylated on the Cdk substrate site), 50 μ g of S100 extract from asynchronous 293 cells, and other indicated components. Crude extracts and T-antigen were preincubated on ice for 30 min with various GST fusion proteins without plasmid DNA, then replication reactions were done by mixing with plasmid DNA and incubation at 37 °C for 1 h.

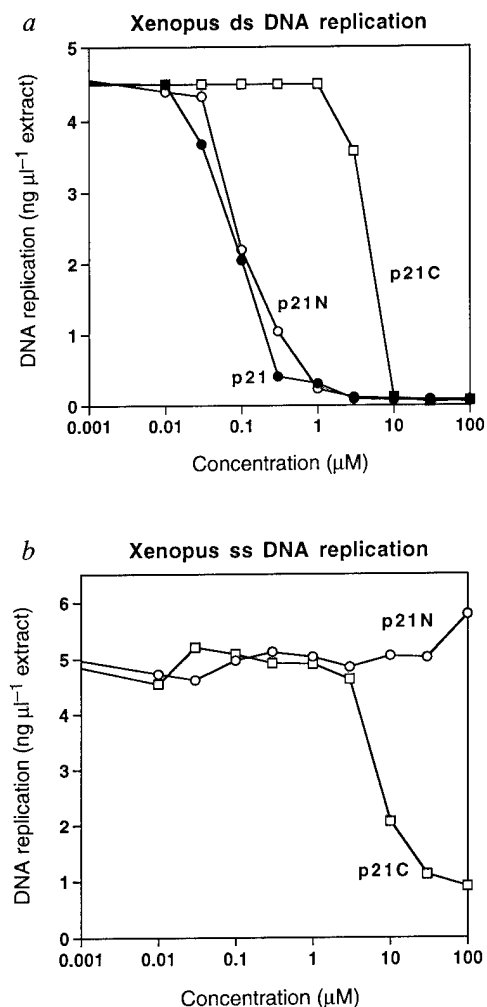


FIG. 3 **a**, p21N inhibits double-stranded sperm DNA replication in *Xenopus* egg extracts at similar concentrations as full-length p21. p21C inhibits DNA replication only when added at concentrations approaching that of PCNA in the *Xenopus* extracts. **b**, p21C but not p21N inhibits DNA synthesis on single-stranded DNA at concentrations similar to that required to inhibit DNA replication on double-stranded DNA.

METHODS. Interphase extracts were prepared from activated *Xenopus* eggs as described (P.K.J. *et al.*, manuscript submitted). Briefly, 10 μ l reactions containing 5 ng μl^{-1} sperm DNA (or single-stranded M13 DNA) and [α -³²P]dATP were mixed with varying concentrations of bacterially expressed GST-fusion proteins, incubated for 2.5 h at 23 °C and assayed for TCA-precipitable counts. Amount of DNA replicated was calculated using the known specific activity of dATP in the reaction.

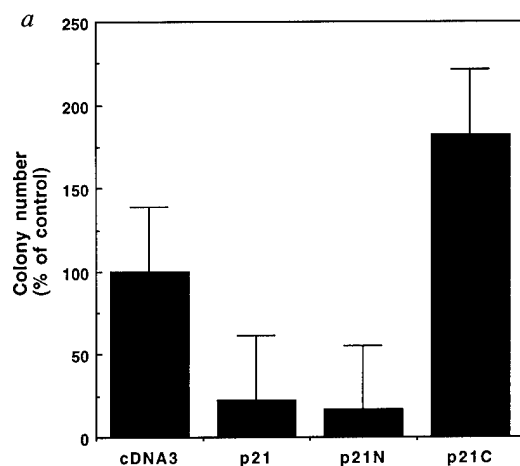
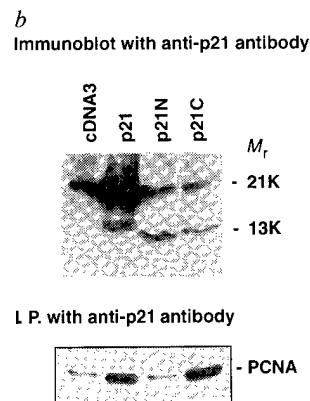


FIG. 4 a, p21N inhibits cell growth whereas p21C does not. Number of G418-resistant colonies obtained after transfection of SaOs2 cells with equal amounts of indicated plasmids is shown relative to the number obtained with vector control alone (cDNA3). Mean and standard deviation of five independent transfections. b, Upper, immunoblot probed with anti-p21 polyclonal antibody shows p21 and its derivatives were expressed *in vivo*. The 21K polypeptide seen in all lanes could be monkey p21. Exogenous proteins of 21, 13 and 13K are also seen in cells transfected with p21, p21N and p21C, respectively. Lower, the C-terminal half of p21 formed a complex with PCNA *in vivo*. Transiently transfected cell lysates were immunoprecipitated with anti-p21 polyclonal antibody and the precipitate immunoblotted with anti-PCNA antibody. All lanes contain PCNA presumably co-immunoprecipitated with monkey p21. Cells expressing p21 and p21C co-immunoprecipitate larger quantities of PCNA because of

plasmids expressing p21 established fewer colonies compared to control vector plasmids³. The N-terminal cyclin-Cdk2 inhibitory domain (p21N) also suppressed growth, and the C-terminal PCNA inhibitory domain (p21C) did not, suggesting that cyclin-Cdks are the primary targets of p21 in transformed cells. An immunoblot of extracts of transfected COS cells shows that human p21, p21N and p21C are expressed from these plasmids and that the exogenous human p21 and p21C proteins associated with and co-immunoprecipitated cellular PCNA (Fig. 4b). The PCNA-interacting portion of p21 (p21C) reproducibly stimulated the number of colonies obtained upon transfection, but the significance of this is unclear.

Having shown that the inhibition of cyclin-Cdk and inhibition of PCNA are independently executed by two different domains of p21, we could now determine which target mediates the effect of p21 in each biological system. Cdk kinases are the primary target for inhibition of double-stranded DNA replication in *Xenopus* extracts by p21 and for growth suppression in transformed cells. In contrast, PCNA is the limiting target in the SV40 replication reaction. The differences between the various systems suggest potential Cdk substrates for activation of DNA replication at the G1-S transition. The insensitivity of SV40 replication of p21N is paradoxical in view of the stimulation of replication in G1 extracts by Cdk kinase^{10,11}. The reported stimulation by Cdk kinase was not mediated through the phosphorylation of T antigen, because those experiments (like the



association of PCNA with the exogenous protein.

METHODS. a, SaOs2 cells (p53 null human osteosarcoma cells) were transfected with 5 µg of the indicated plasmid and 10 µg of salmon sperm carrier DNA by the calcium-phosphate method¹¹. After 14–21 days of selection with G418 at 400 µg ml⁻¹ the number of colonies per transfection were counted. cDNA3 (Invitrogen) is the control vector which expresses genes inserted downstream from a cytomegalovirus promoter and contains a neomycin phosphotransferase gene. p21, p21N and p21C plasmids were made by transferring *Bam*HI–*Xho*I fragments from the corresponding pGEX 5X-3-derived plasmids (Fig. 1) into the same sites of cDNA3. b, COS cells (T-antigen transformed monkey kidney epithelial cells) were transfected as above with the indicated plasmids and collected 60 h later. Cell lysis and immunoprecipitation were done as previously described¹⁶. For the upper and lower panels, respectively, 110 and 1,100 µg of each cell lysate was used.

ones reported here) used T antigen which was already phosphorylated on the Cdk substrate site, threonine 124. One explanation could be that Cdk kinase is required specifically to overcome G1-specific factors and hence is dispensable for replication in extracts from asynchronous cells. In *Xenopus* extracts, potential targets for Cdk2 could be the hypothetical G1-specific factors, chromatin or origin-binding proteins (the T-antigen equivalent). Also, the failure of p21N to inhibit DNA synthesis on single-stranded DNA in *Xenopus* extracts suggests that Cdks are primarily required for the unwinding of and initiation of DNA synthesis on double-stranded DNA.

Inhibition of PCNA by p21 (and p21C) is not apparently required in the particular growth suppression assay used in this report^{6,12,13}. Transformed cells may have an excess of PCNA, so that higher levels of p21C are required to inhibit PCNA function. Inhibition of PCNA may also be more important for the p21-mediated temporary switch from DNA replication to repair following genotoxic damage¹². The ability of p21N alone to inhibit cell-cycle progression has interesting implications for the activity of other Cdk-inhibitory and cell-cycle blocking polypeptides like p27 (upregulated by transforming growth factor-β (TGF-β)) which show homology with p21 in the region contained in p21N^{14,15}. If the Cdk2-interacting domain of p21 can be narrowed down further, it may be possible to design small molecules that mimic the structure of this peptide. Such chemicals could suppress growth of cancers with loss of functional p53 or potentiate the growth-suppressive effect of TGF-β. □

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A 39 amino acid fragment of the cell cycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA replication *in vivo*

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ABSTRACT

The cell cycle regulator p21 interacts with and inhibits the DNA replication and repair factor proliferating cell nuclear antigen (PCNA). We have defined a 39 amino acid fragment of p21 which is sufficient to bind PCNA with high affinity (K_d 10–20 nM). This peptide can inhibit DNA replication *in vitro* and microinjection of a GST fusion protein containing this domain inhibited S phase *in vivo*. Despite its high affinity for PCNA, the free 39 amino acid peptide does not have a well-defined structure, as judged from circular dichroism and nuclear magnetic resonance measurements, suggesting an induced fit mechanism for the PCNA–p21 interaction. The association of the small peptide with PCNA was thermolabile, suggesting that portions of p21 adjoining the minimal region of contact stabilize the interaction. In addition, a domain containing 67 amino acids from the N-terminus of PCNA was defined as both necessary and sufficient for binding to p21.

INTRODUCTION

Normal cell cycle progression involves a sequential increase in the levels of various cyclins, their association with corresponding cyclin-dependent kinases (cdk) and sequential activation of these kinase activities in the different phases of the cycle. Cyclins, cdk kinases, the cdk inhibitor p21 and the DNA replication factor proliferating cell nuclear antigen (PCNA) have been found to form a quaternary complex in untransformed cells (1–3). Besides associating with and inhibiting cdk2 kinase (4–8), p21 has an additional activity through its interaction with the DNA replication factor PCNA. PCNA is an auxiliary factor for DNA polymerases δ and ϵ and is essential for DNA replication *in vitro* and *in vivo* (9–15). p21 interacts with PCNA and inhibits its activity (16–21). p21 is transcriptionally induced by the tumor suppressor protein p53, which is itself increased in response to DNA damage, and it

has been suggested that the p21 is an important effector of the growth suppressive function of p53 (22).

We and others have reported that the N-terminal 90 amino acids of p21 inhibited cyclin–cdk kinase activity, DNA replication in *Xenopus* egg extracts and cell growth in p53 null-transformed cancer cell lines (19,23–25). The C-terminal 77 amino acids of p21 interacted with PCNA and inhibited SV40-based DNA replication and *Xenopus* DNA replication. A small chemical based on the structure of p21 which interacts with and inhibits PCNA could be useful for suppressing cell growth or for inhibiting DNA repair after radio- or chemotherapy. We have determined that peptides based on the structure of p21 suppress cell growth when delivered *in vivo* at high concentrations and have measured the K_d of the peptide–PCNA interaction to determine if it was suitable for pharmacology. Despite the high affinity of the peptide–PCNA interaction, circular dichroism and NMR studies show that the free peptide is flexible in structure, suggesting an induced fit mechanism for the peptide–PCNA interaction. We also demonstrate that the N-terminal 67 residues of PCNA are necessary and sufficient for the interaction and that there are multiple potential binding sites for p21 on each PCNA trimer. Taken together these results indicate that while a peptide derived from p21 may itself be unsuitable for targeting the DNA replication and repair apparatus, a synthetic chemical based on the structure of the PCNA-bound peptide could be effective *in vivo*.

MATERIALS AND METHODS

Plasmids

pGST-p21, pGST-p21N and pGST-p21C were generated as described (23). pGST-p21M1 and pGST-p21C2 were generated by PCR with *Pfu* polymerase and cloned into *Bam*HI and *Sal*I sites of pGEX-5X3 (Pharmacia). pETPCNA has been described (26).

Protein expression and purification

Bacterially produced proteins were expressed in *Escherichia coli* BL21. Protein induction, cell lysis and affinity purification with

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glutathione-agarose beads were as described (27). *In vitro* transcription-translation reactions were as suggested by the manufacturer (Promega).

Synthesis of peptides

A 41 amino acid p21C2 peptide (consisting of the 39 C-terminal amino acids of p21 plus two Lys residues at the C-terminal end required for chemical synthesis) was synthesized at the Harvard Medical School Biopolymer Laboratory using a Milligen/Bioscience 9600 synthesizer. The peptide was purified using C18 reverse phase HPLC.

The sequences of peptides used were:

p21C2:QAEGSPGGPGDSQGRKRRQTSMTDFYHSKRRLIFSRRKPKK;
 CSH262:WNSGFESYGSSSYGGAGGYTQAPGGFGAPAPS-QAEKKSRR;
 CSH119:ADAQHAAPPKKRKRVEDPKDF.

Assays

Affinity chromatography on glutathione beads coated with various GST fusion proteins ('pull-down' assays) was as described (23,27), except that washes were with buffer A7.4 (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.01% NP-40, 10% glycerol, 25 mM NaCl). Unlabeled proteins were detected by immunoblotting with appropriate antibodies and ECL reactions. Proteins produced by *in vitro* transcription-translation were labeled with [³⁵S]methionine and visualized by fluorography.

The SV40 DNA replication reaction was performed as previously described (23,28). Aliquots of 180 ng pSV011 were replicated in a 30 μ l reaction containing 100 ng T antigen and 50 μ g S100 extract from cell cycle asynchronous 293 cells. Cell extracts and T antigen were pre-incubated on ice for 30 min with GST fusion proteins or peptides without plasmid DNA, then replication reactions were performed by mixing plasmid DNA and incubation at 37°C for 1 h.

Gel filtration

Protein or protein mixtures were incubated on ice for 15 min in A7.4 buffer before loading onto a 25 ml Superose 12 gel filtration column (Pharmacia). Proteins were eluted from the column at a flow rate of 0.4 ml/min. Fractions of 0.5 ml were collected, separated by 15% SDS-PAGE and stained with Coomassie blue to visualize the proteins PCNA (37 kDa), Fen1 (45 kDa) and p21C2 (4.2 kDa).

Scatchard analysis

Bacterially expressed human PCNA was purified as described (26) and labeled with ¹²⁵I using Bolton-Hunter reagent and following the manufacturer's instructions (Du Pont). Varying amounts of GST-p21C or GST-p21C2 (at least a 30-fold molar excess compared with PCNA) were incubated with a fixed amount of radiolabeled PCNA for 1 h at 4°C or for 15 min at 37°C in buffer A7.4. The GST proteins were recovered by binding to glutathione-agarose beads and the amount of bound PCNA estimated by counting in a gamma counter. All points on the Scatchard plots are the result of at least four different binding assays done on at least two separate days. Care was taken to subtract non-specific binding to GST beads.

The data was analyzed by Scatchard plot according to the equation

$$b/R_t = -b/K_d + B_{\max}/K_d$$

where b is the concentration of bound PCNA (in c.p.m./200 μ l), R_t is the total concentration of GST fusion protein (in nM), B_{\max} is the concentration of total PCNA that can be bound by the GST fusion protein (c.p.m./200 μ l) and K_d is the dissociation constant (in nM). K_d was estimated from the slope of the graph of b/R_t versus b (29).

Microinjection

IMR90 human diploid fibroblast monolayers growing on glass coverslips (at 60% density) were synchronized in G0 by serum starvation for 48 h and stimulated to enter G1 by addition of 10% fetal bovine serum. Fifteen hours after re-activation cells in G1 were microinjected with the indicated proteins using an automated microinjection system (AIS; Zeiss). All microinjection experiments were carried out in 3.5 cm Petri dishes containing 3 ml carbonate-free DMEM, in order to avoid a decrease in pH of the medium during the injection. Each cell was injected with protein or peptide (3.75 mg/ml in PBS) together with normal rabbit immunoglobulin (2.5 mg/ml) at a pressure between 50 and 150 hPa. The computer settings for injection were angle '45', speed '10' and time of injection '0.0 s', so as to deliver 0.01–0.05 μ l liquid/nucleus. For more details of the microinjection procedure see Pepperkok (30).

DNA synthesis was monitored by incubating with BrdU (100 μ M; Amersham) for 10–12 h after microinjection. Coverslips were then rinsed in PBS and fixed for 10 min in -20°C cold methanol/acetone (1:1) and washed again three times with PBS. Microinjected cells were detected by incubation for 1 h with biotinylated horse anti-rabbit IgG (diluted 1:50; Vector Laboratories), washed three times with PBS and incubated with Texas red-conjugated streptavidin (diluted 1:100; Vector Laboratories). Coverslips were subsequently incubated for 10 min with 1.5 M HCl, washed three times with PBS and then incubated for 1 h with a solution of mouse monoclonal anti-BrdU antibody plus nuclease (undiluted; Amersham), followed by a 30 min incubation with a 1:50 dilution of an anti-mouse FITC-conjugated antibody (Vector Laboratories).

All antibody reactions were carried out in a humidified chamber at room temperature and dilutions were made in DMEM containing 10% FCS. Counterstaining for DNA was performed by adding 1 μ g/ml bisbenzimidazole (Hoechst 33258) to the final PBS wash. Immunofluorescence samples were directly mounted in Crystal/mount medium (Biomed Corp.). Photographs were taken using a Plan-Neofluar 40 \times lens mounted on a Zeiss Axiophot Photomicroscope and a Color Video Printer Mavigraph on Sony UPC-3010 print paper.

In each experiment ~100 injected cells (and a corresponding number of non-injected cells) were counted. Per cent inhibition of BrdU incorporation was calculated as $[(N - I)/N] \times 100$, where N is percentage BrdU incorporation in non-injected cells and I is percentage BrdU incorporation in cells microinjected with antibodies. The obtained numerical value is independent of possible experimental variations in the number of BrdU-positive cells that had not been injected.

Circular dichroism

Spectra were obtained at a concentration of 22 μ M (p21C2) in PBS, pH 7.0. A path length of 0.1 cm in an Aviv 62DS spectropolarimeter equipped with a temperature control unit was used. Spectra were obtained with a scan speed of 1 s at each wavelength. Mean residue

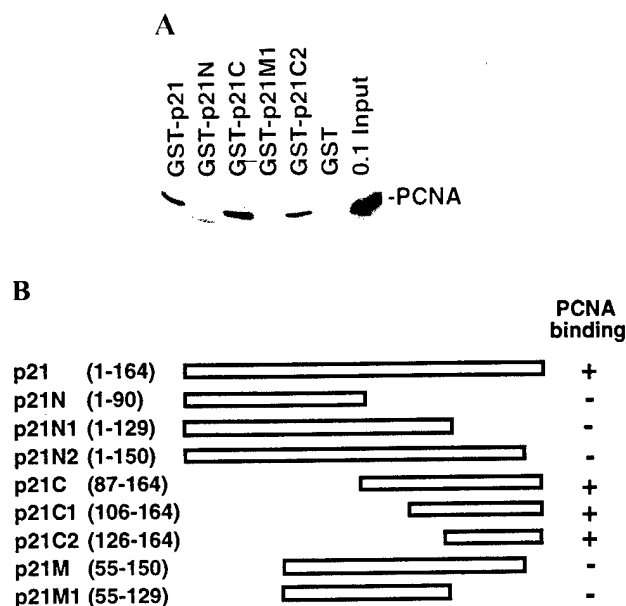


Figure 1. Deletion analysis of p21 shows that the C-terminal 39 amino acids are sufficient for binding PCNA. (A) Immunoblot with anti-PCNA antibody. The indicated GST fusion proteins were used to mediate the binding of bacterially produced human PCNA (37 kDa) to glutathione-agarose beads. One tenth of input PCNA is shown for comparison. The smaller band seen in the second lane is the GST-p21N protein, which is ~35 kDa in size. Due to their high protein content, GST fusion proteins produce background bands in the enhanced chemiluminescence reaction used to visualize the immunoblots. (B) Schematic summary of deletion derivatives of p21 and their ability to bind PCNA [(A) and data not shown]. The numbers indicate which amino acids of p21 are present in the deletion derivatives.

ellipticity (θ) was calculated with a calculated molecular weight of 4562 g/m.

NMR spectra

All experiments were run on a Varian VXR500 spectrometer. Spectra were recorded at 2 mM sample concentration in PBS, 10% D₂O, pH 7.0. NOESY spectra were recorded at 5 and 25°C with mixing times of 150 and 300 ms. TOCSY spectra were recorded at 25°C with mixing times of 50 and 75 ms.

RESULTS

The C-terminal 39 amino acids of p21 are sufficient to interact with PCNA

Bacterially expressed glutathione S-transferase-p21 (GST-p21), GST-p21C and GST-p21C2 were used as an affinity matrix to demonstrate that PCNA interacts with the last 39 amino acids of p21 (Fig. 1 and data now shown). Scatchard analysis of the interaction (at 4°C) showed that the K_d values for the GST-p21C-PCNA and GST-p21C2-PCNA interactions were 15.4 and 12.0 nM respectively (Fig. 2). At 37°C the K_d of the GST-p21C-PCNA interaction was unchanged, but that of GST-p21C2-PCNA increased 100-fold. A synthetic 41 amino acid peptide corresponding to p21C2 (plus two lysines at the C-terminus) was synthesized. In agreement with the K_d measurements, the synthetic peptide competitively inhibited binding of PCNA to GST-p21 at 4°C (Fig. 3a), but failed to compete with GST-p21 for binding to PCNA at 37°C (Fig. 3b).

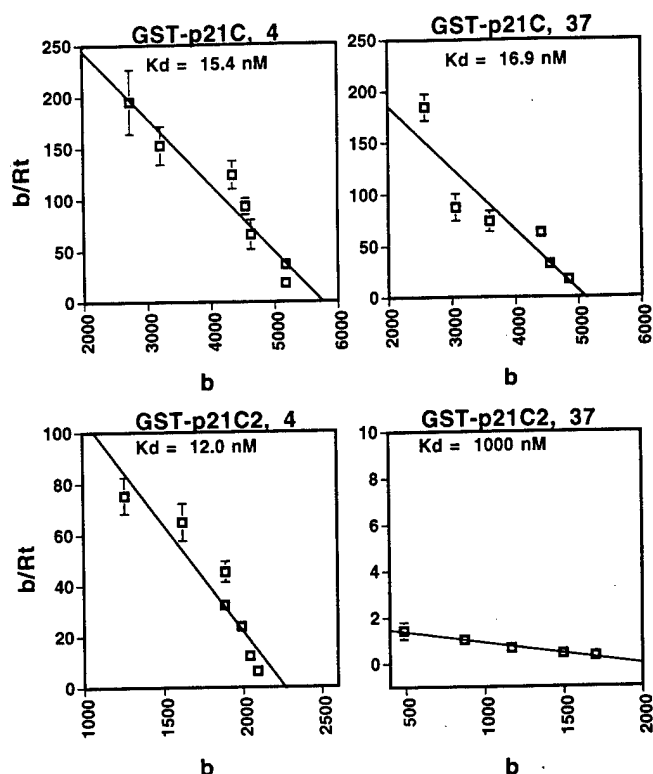


Figure 2. Scatchard analysis of the binding of PCNA to GST-p21C or GST-p21C2 at 4 or 37°C. The y-axis shows the ratio of bound PCNA (c.p.m./200 μ l) to the total concentration of the GST fusion protein (nM) (b/R_t). The x-axis shows the amount of bound PCNA (c.p.m./200 μ l) (b). Each point is the mean \pm SD of four measurements and the slope of the line equals $-1/K_d$.

These experiments demonstrate that the C-terminal 39 amino acids of p21 are sufficient to bind PCNA. However, an additional 38 amino acids (present in GST-p21C but not in GST-p21C2) stabilize the interaction and prevent loss of affinity as the temperature is increased to the physiological range.

Inhibition of the SV40 replication reaction

Since the interaction of p21 with PCNA inactivates its function as a DNA replication factor, we measured the abilities of the GST fusion proteins to inhibit the SV40-based DNA replication reaction (Fig. 4). The concentration required to obtain 50% inhibition of replication (IC_{50}) was 0.5–1 μ M for GST-p21 or GST-p21C and 9 μ M for GST-p21C2. The synthetic p21C2 peptide was slightly weaker than GST-p21C2 at inhibiting SV40 replication (IC_{50} 14 μ M), but addition of 1% DMSO to the replication reaction improved inhibition by the p21C2 peptide ~2-fold (data not shown). The 10- to 20-fold weaker inhibitory activity of GST-p21C2 compared with GST-p21C could be consistent with its lower affinity for PCNA at 37°C. Inhibition of DNA replication by p21C2 was reversed by addition of excess PCNA (data not shown). We tested whether amino acids 87–125 of p21 (present in p21C, but not in p21C2) contributed to inhibition of SV40 DNA replication by interacting with and inhibiting a second replication factor. A fragment of p21 containing this region, GST-p21M1, was unable to bind PCNA (Fig. 1) or inhibit the DNA replication reaction (Fig. 4). These results suggest that amino acids 87–125 of p21 contribute to replication inhibition only

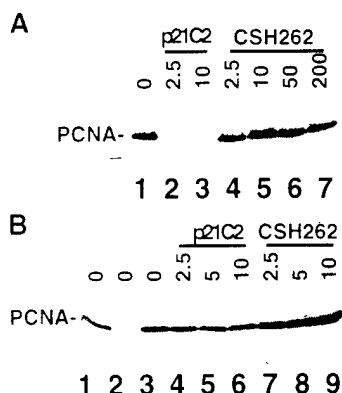


Figure 3. Synthetic p21C2 peptide can competitively inhibit p21-PCNA interaction at 4 but not at 37°C. Binding of PCNA visualized by immunoblotting of bead-bound proteins with anti-PCNA antibody. (A) 1 μM GST-p21 was incubated at 4°C with 100 μg S100 extract from 293 cells. Concentrations (μM) of peptide competitor are indicated at the top: p21C2 peptide (lanes 2 and 3) or negative control peptide CSH262 (lanes 4–7). (B) As (A) except the reaction was carried out at 37°C. Lane 1, one tenth input lysate; lane 2, bound to GST protein; lanes 3–9, bound to 1 μM GST-p21 protein. Competing peptides were none (lanes 1–3) or the indicated concentrations of p21C2 or negative control peptide CSH262.

by stabilizing the p21-PCNA interaction. However, the 39 amino acid region of p21 was still an effective inhibitor of DNA replication *in vitro*.

Effect of GST-p21C and p21C2 peptides on entry of quiescent cells into S phase

To determine whether a p21-based peptide was active *in vivo* at reaching and interacting with PCNA we analyzed whether S phase was inhibited by these proteins. Quiescent diploid fibroblasts were stimulated by serum and entry into S phase followed after microinjection of GST fusion proteins or the p21C2 peptide (Fig. 5). GST-p21, GST-p21N and GST-p21C inhibited uptake of BrdU significantly compared with a negative control peptide CSH119, GST alone or GST fused to the cell cycle regulatory protein cdc25C (31). Thus GST-p21C inhibits growth of cells almost as well as GST-p21N when provided in high enough concentrations. Consistent with the result from the *in vitro* SV40 replication reaction, GST-p21C2 inhibited entry into S phase, although less effectively than GST-p21C. Surprisingly, the p21C2 peptide was only a weak inhibitor of cell growth. The difference between GST-p21C2 and the p21C2 peptide was observed consistently and was statistically significant ($P < 0.05$ by ANOVA). The results also confirm earlier reports that p21N, which binds and inhibits cdk kinases but not PCNA, inhibits growth of cells almost as effectively as p21.

Deletion mapping the part of PCNA which binds p21

Full-length PCNA and various deletion derivatives were synthesized by *in vitro* transcription-translation and binding to GST-p21 measured in a pull-down assay (Fig. 6). Since full-length PCNA bound to p21 well but a fragment of PCNA containing residues 40 to the C-terminus (40–C) did not, it appeared that the N-terminal portion of PCNA was important for binding p21. Consistent with this possibility, derivatives of PCNA containing amino acids 1–127

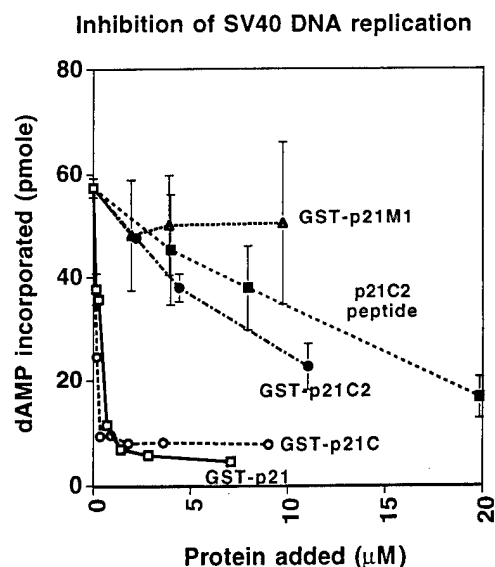


Figure 4. Inhibition of SV40 DNA replication by fragments of p21. The proteins added were GST-p21 (open squares), GST-p21C (open circles), GST-p21C2 (closed circles), GST-p21M1 (open triangles) and p21C2 peptide (closed squares). Each point represents the mean \pm SD of three separate measurements of DNA replication (amount of dAMP incorporated into polynucleotide).

and 1–67 bound to p21. We conclude that a p21 binding domain of PCNA resides in the N-terminal 67 amino acids, perhaps even in the N-terminal 40 residues. The 1–127 fragment could have interacted with p21 indirectly as part of a larger complex with a protein present in the reticulocyte lysate (e.g. full-length rabbit PCNA). To test if this was the case the *in vitro* translation mix was fractionated by glycerol gradient sedimentation. The 'light' fractions, where the 1–127 fragment sediments in the same position as cytochrome c (and much lighter than the position of endogenous PCNA), could still associate with p21 (data not shown). Therefore, it is likely that the isolated 1–127 fragment of PCNA associates directly with p21.

The stoichiometry of the p21-PCNA interaction has been reported as 1:1 (p21 to trimer) (16) or 2.3:1 (18). Our observation that an isolated part of a PCNA monomer binds to p21 suggests that there could be more than one p21 binding site per PCNA trimer. p21C2 peptide was mixed with PCNA trimers at different ratios and subjected to gel filtration (Fig. 7). Even when p21C2 peptide was added at a ratio of 6 molecules peptide/PCNA trimer all the peptide was bound to PCNA and co-eluted with alcohol dehydrogenase (150 kDa). As a negative control p21C2 was mixed with another DNA replication/repair factor, Fen1, and subjected to gel filtration. All of the peptide eluted from the column after cytochrome c (14 kDa). The position of elution indicates that the p21C2 peptide is not present as a hexamer (30 kDa). Glycerol gradient sedimentation of 6-histidine-tagged p21 also indicates that the molecule exists as a monomer (16). Therefore, the association of virtually all the p21C2 peptide with PCNA even at a ratio of 6 peptide molecules/PCNA trimer is consistent with the model that there are multiple p21 binding sites per PCNA trimer (18). Even though there may be six potential binding sites for p21C2 peptide per PCNA trimer we favor a model where three molecules of p21 bind per PCNA trimer (see Discussion).

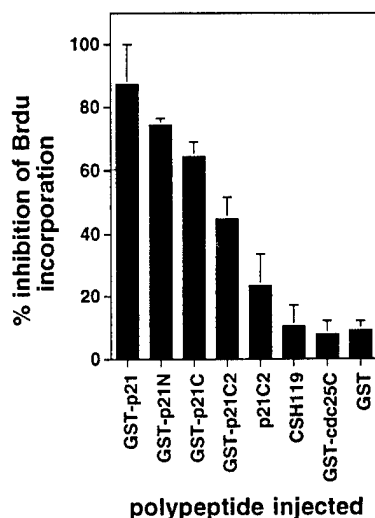


Figure 5. Inhibition of entry into S phase by microinjection of GST-p21 fusion proteins and indicated peptides into nuclei of serum re-activated diploid fibroblasts 15 h after re-activation. Mean \pm SD for at least three different experiments are shown. CSH119, GST and GST-CDC25C were the negative controls, with indicated growth inhibition probably being a side effect of the injection procedure.

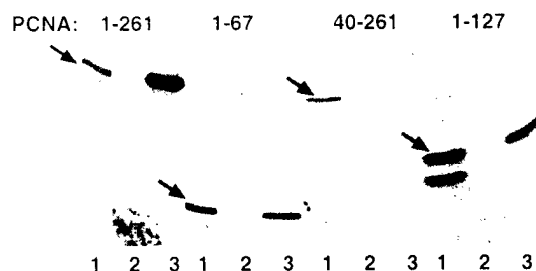


Figure 6. Deletion mapping of the part of PCNA that binds to p21. Full-length (1-261) or fragments containing the indicated residues of PCNA were produced by *in vitro* transcription-translation and visualized by fluorography. Lane 1, one tenth input; lane 2, protein bound to GST-coated beads; lane 3, protein bound to GST-p21-coated beads. Arrows indicate the PCNA (full-length or deletion derivative) in the input lane of each set.

Secondary structure of p21C2 peptide by circular dichroism and nuclear magnetic resonance

In view of the high affinity with which GST-p21C2 binds PCNA and inhibits S phase *in vivo*, a structural analog of the p21C2 domain would be a strong candidate for pharmacological use. Since the p21C2 peptide bound PCNA well at 4°C, we attempted to determine its structure.

The structure of the synthetic peptide p21C2 was studied by circular dichroism. A representative spectrum is shown in Figure 8. The peptide does not appear to have a well-defined secondary structure. The spectrum displays a minimum at 200 nm and a maximum at 220 nm (32). Temperature dependence of the CD spectra was monitored at 200 and 220 nm (Fig. 8, inset) and failed to show any appreciable change in ellipticity with change in temperature, confirming the lack of folded structure.

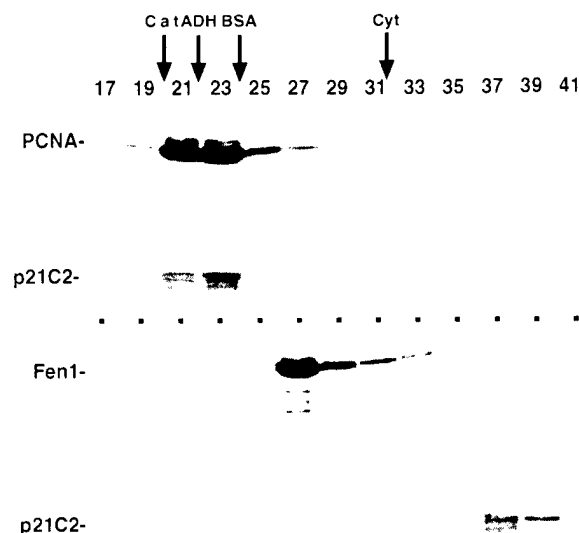


Figure 7. Gel filtration analysis of complexes formed by p21C2 peptide with PCNA (top) and Fen1 (bottom). Peptide and PCNA (molar ratio 2:1, equal to 6 molecules peptide/PCNA trimer) or peptide and Fen1 (molar ratio 2:1) were incubated and the complexes analyzed by chromatography on a Superose12 column. Alternate fractions were visualized by SDS-PAGE and Coomassie blue staining. The positions of elution of molecular weight markers is indicated by arrows at the top: catalase (Cat, 240 kDa), alcohol dehydrogenase (ADH, 150 kDa), bovine serum albumin (BSA, 66 kDa) and cytochrome c (Cyt, 12.5 kDa).

To further investigate the structure of the peptide ^1H NMR experiments were run in aqueous conditions. The NOESY spectrum (Fig. 9) lacks any significant number of inter-residue cross-peaks. The only cross-peaks seen are intra-residue, between amide protons and α protons (Fig. 9c) or side chain protons (Fig. 9d) of the same residue or sequentially adjacent residues. In particular, amide-amide cross-peaks are absent, which would be characteristic of an organized protein structure (Fig. 9a). Furthermore, the chemical shift values of each amino acid determined by TOCSY experiments are identical (within experimental error) to published random coil ^1H chemical shift values (data not shown) (33).

Altogether, the above spectroscopy results (circular dichroism and NMR), which did not vary under a large variety of aqueous conditions (temperature, buffer and pH; data not shown), demonstrate that unbound p21C2 does not adopt a well-defined structure in an aqueous environment.

DISCUSSION

The DNA replication enzymes are attractive targets for development of new agents for chemotherapy (34). We examined the p21-PCNA interaction with the long-term goal of determining if it could be exploited for the design of drugs which reach their target (PCNA) *in vivo*. As a first approximation we used a peptide (p21C2) derived from p21 which interacted with PCNA and inhibited the SV40 replication reaction *in vitro*. A 10-fold higher concentration of GST-p21C2 or the free p21C2 peptide was required to inhibit the SV40 replication reaction compared with GST-p21C. This is likely to be due to the 100-fold decrease in affinity of p21C2 for PCNA at physiological temperatures, although we cannot rule out the existence of factors in cell extracts that specifically interfere with the action of p21C2, but not p21C.

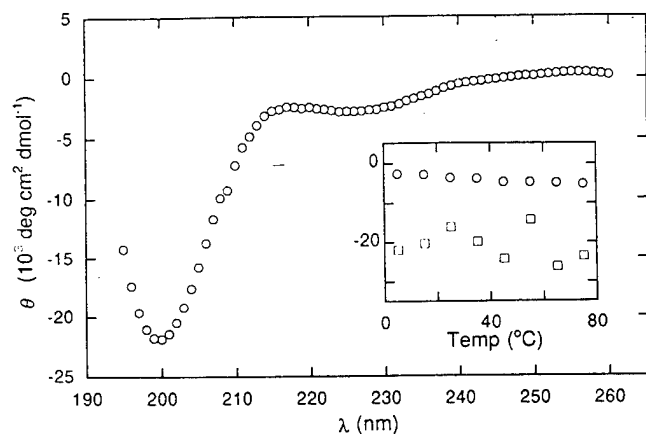


Figure 8. Far-UV circular dichroism spectrum of the p21C2 peptide in PBS (pH 7) at 4°C. (Inset) Effect of temperature on ellipticity measured at 220 nm (squares) and 200 nm (circles).

These results agree with a recent report that a 20 amino acid peptide from the C-terminal part of p21 (141–160) binds and inhibits PCNA *in vitro* (35). Point mutations have also indicated that multiple amino acids in this same region of p21 and additional ones at residues 161–163 are crucial for interaction with PCNA (36).

The efficacy of p21-based peptides at reaching and inhibiting PCNA *in vivo* was not clear before the present study. Because GST-p21C2 effectively inhibited cell growth but the free p21C2 peptide did not, we suspect that smaller peptides are unlikely to be useful in inhibiting PCNA *in vivo*. However, the high affinity of the interaction between GST-p21C and PCNA (K_d 10–20 nM) suggests that this interaction is suitable for pharmacological purposes. For comparison, other protein–protein interactions which have the potential for development as therapeutic agents include inhibition of cyclin–cdk kinases by p21 (K_i 1 nM) (37), interaction between phosphotyrosine-containing peptides and SH2 domains (K_d 10–100 nM) (38,39) and interaction between SH3 domains and proline-rich peptides (K_d 1000 nM) (40).

In general, peptide-based therapeutic agents suffer from the obvious problem of delivering peptides into cells at high concentrations. Our results point to two additional drawbacks: decreasing the length of the interacting peptide rendered the interaction thermodynamically unstable and additional poorly understood mechanisms were responsible for the small p21C2 peptide, but not GST-p21C2 protein, being inactivated in the cell. A small chemical that can mimic the structure of the active PCNA binding region of p21C2 may overcome all these drawbacks. Such a chemical may also be used to target other replication inhibitors to the site of DNA synthesis. Therefore, the best approach will be to determine the structure of the p21C2 binding interface and design chemicals which mimic this. Results reported in this paper indicate that the free p21C2 peptide lacks organized structure, suggesting that one has to determine the structure of the PCNA-bound peptide for this purpose.

Differences in the relative intracellular concentrations of p21C probably explain why S phase is inhibited by microinjection of GST-p21C, while transfection of plasmids expressing p21C failed to inhibit colony formation in an earlier assay (23,24). Expression from transfected plasmids is unlikely to yield as high a concentration of p21C per nucleus as is obtained by microinjection. Quiescent diploid fibroblasts have very little PCNA and as they enter the cell

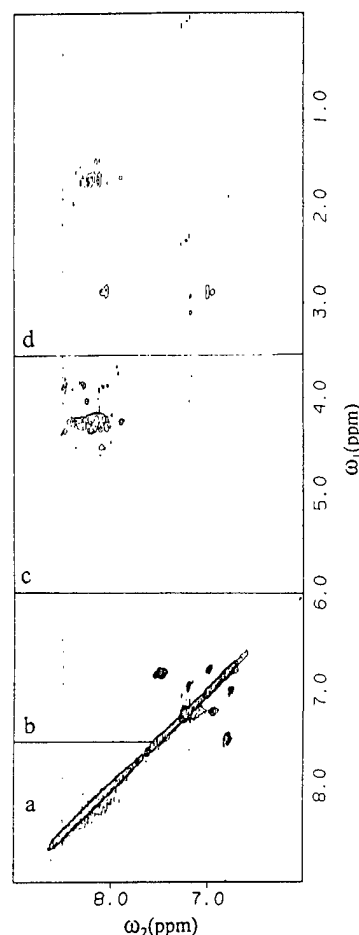


Figure 9. ^1H NOESY spectrum of p21C2 peptide in PBS at 25°C with a 150 ms mixing time. The low field half (ω_2 6.0–9.0 p.p.m.) of the spectrum is shown. Above the diagonal the spectrum is divided into four regions (a–d) where cross-peaks between distinct proton types occur (see text).

cycle new PCNA has to be synthesized to support DNA replication. Therefore, the low levels of PCNA and the fact that new PCNA is not sequestered in replication complexes are additional factors which favor cell growth suppression by p21C in the experiments reported here.

Using the two-hybrid method of studying protein–protein interactions another group has studied the domain of PCNA that interacts with p21 (35). A series of progressively increasing N-terminal deletions showed that amino acids 50–261 (50-C-terminus) and 100–261 of PCNA could interact with p21, but amino acids 150–261 could not, suggesting the importance of amino acids 100–150 of PCNA in the interaction with p21. Our biochemical method of assaying p21–PCNA interaction fails to show an interaction with the 40–261 derivative of PCNA, yet shows significant interaction of amino acids 1–67 or 1–127 of PCNA with p21. The two-hybrid method uses a version of PCNA with the yeast Gal4 activation domain fused at the N-terminus. Such a fusion may partially denature PCNA and permit interactions not possible with the trimeric PCNA complex. We find that GST-PCNA (where GST protein is fused to the N-terminus of PCNA) does not bind p21 (data not shown), so that the Gal4–PCNA fusion may also have inactivated the N-terminal p21 binding site. Alternatively, each PCNA molecule being composed of two structurally homologous

domains, amino acids 1–67 and 100–150 contain structurally similar N-terminal regions from each of the two domains (41). Therefore, the interaction with p21 could be executed by two structurally homologous regions of PCNA: a strong p21 binding region at the N-terminus and a weak binding region at amino acids 100–150. The weaker binding site in the 40–261 derivative may not be sufficient to give a positive signal in our assay, but could give a signal in the more sensitive two-hybrid assay. If this alternative is correct, each PCNA trimer may have up to six potential binding sites for p21.

The flexible nature of the p21C2 peptide may have been created by deletion of more than 75% of the p21 protein. However, despite the flexible structure, the high affinity and specificity of GST–p21C2 for PCNA at 4°C suggests that p21C2 is induced into a specific conformation when interacting with PCNA. This possibility is also favored by the observation that interaction of the 39 amino acid region is temperature sensitive and that the adjoining 38 amino acids stabilize the interaction (GST–p21C2 versus GST–p21C at 37°C).

Since isolated PCNA monomers and portions thereof bind p21, there are likely to be more than one p21 binding site per PCNA trimer. Are there six binding sites per PCNA trimer? We have provided experimental evidence which suggest that six p21C2 peptides could bind per PCNA trimer. However, it is unlikely that six molecules of p21 could bind to PCNA and not change the sedimentation profile of PCNA (16). Surface plasmon resonance spectroscopy showed that 2.3 molecules of p21 bind per PCNA trimer (18). Therefore, although there may be six sites per PCNA ring for association with p21, only a fraction of these can be occupied simultaneously by a molecule as large as p21. Nevertheless, multiple p21C2 binding sites on each PCNA ring translate into multiple binding sites for a chemical based on the binding interface of the peptide.

In conclusion, these results indicate that the p21–PCNA interaction has properties that may be useful for the design of drugs targeted to the replication fork. The affinity of the interaction is high, as are the number of binding sites per PCNA trimer. GST fusion proteins containing the peptide can interact with PCNA in the cell. However, direct use of peptides based on p21 will not be useful. Instead, one will have to determine the structure of the binding interface of p21 in the p21–PCNA complex and design chemicals based on this structure.

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Loss of transactivation and transrepression function, and not RPA binding, alters growth suppression by p53

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The tumor suppressor protein p53 activates transcription from promoters with specific p53 binding elements, represses transcription from promoters without such elements and interacts with and inhibits the single-stranded DNA binding activity of the human DNA replication factor RPA. All these activities involve the N terminal 70 amino acids of p53. Dissection of the domains of p53 which bind RPA suggest that multiple sub-domains of the protein synergize to give strong RPA binding. Point-mutations in one of these sub-domains of p53 significantly diminish its ability to interact with RPA. A multimer of a peptide from p53 which includes these residues, or of a peptide from the acidic activation domain of the prototypic trans-activator protein VP16, can itself bind to RPA. Comparison of sequences of these multimeric peptides suggests that aromatic amino acids flanked by negatively charged residues are important for binding RPA. Several alleles of p53 with point mutations in the N terminal region were analysed for their relative abilities to bind RPA, activate or repress transcription, and suppress growth of p53 null SaOs2 and H1299 cells. Both mutants of p53 with decreased RPA binding suppressed cell growth as well as wild-type p53, suggesting that p53 can suppress growth without interacting with RPA. The allele that lost most of the transcription activation function also lost most of its transcription repression activity suggesting that interaction with the same basal transcription factors are involved in both functions. This same allele bound RPA well but was defective in growth suppression. Therefore, transcription activation and/or repression appear to be more important for the growth suppression function of p53 than RPA binding.

Keywords: p53; RPA; transcription; cell cycle; DNA replication

Introduction

Since its discovery in 1979, many investigators have convincingly demonstrated that p53 has a critical role in the cell. By halting abnormal cell division, p53 can suppress the uncontrolled growth that leads to neoplasia. Overexpression of the wild type p53 protein arrests cell growth just before the onset of DNA replication at the G1-S boundary. Wild type p53 is essential for G1 arrest following radiation-induced

DNA damage, or for apoptosis of the cell if the DNA damage is extensive (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). Also, wild type p53 suppresses the potential of a cell to amplify portions of its genome (Livingstone *et al.*, 1992; Yin *et al.*, 1992). The transforming mutants of p53 are defective in all these functions. Therefore a major concern in the field of cancer research is how the wild type p53 protein carries out these diverse functions: inhibition of S phase, pause in DNA replication following DNA damage, induction of apoptosis and inhibition of DNA amplification.

Three mechanisms have been proposed by which p53 inhibits DNA replication. First, p53 could act as a suppressor of S phase by the sequence specific transcriptional induction of genes (Fields *et al.*, 1990; Raycroft *et al.*, 1990; Pietenpol *et al.*, 1994) that negatively regulate cell growth e.g. the p21 gene (El *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Noda *et al.*, 1994). Indeed, several authors to date have found a correlation between transcription activity and p53's ability to suppress growth (Crook *et al.*, 1994; Pietenpol *et al.*, 1994). Second, p53 can more generally repress transcription from certain cellular promoters (Seto *et al.*, 1992; Mack *et al.*, 1993; Crook *et al.*, 1994; Subler *et al.*, 1994). Since these promoters do not contain p53 binding sites, it is thought that p53 may reduce transcription by binding to and sequestering basal transcription factors. The role of p53's trans-repression activity in growth suppression is less consistent in the literature, however. Third, p53 interacts with the SV40 T antigen, inhibits the helicase activity of T antigen and inhibits the SV40 based *in vitro* DNA replication (Friedman *et al.*, 1990). Therefore p53 could potentially interact with and inhibit a cellular origin binding replication initiator protein or replication helicase (as yet unidentified). This is supported by the recent report by Cox *et al.* (1995) that p53 inhibited nuclear DNA replication in a transcription-free DNA replication extract from *Xenopus* eggs.

We and others reported that p53 interacts with a cellular DNA replication factor RPA (Dutta *et al.*, 1993; Li *et al.*, 1993). RPA (RF-A or human ssb) is a complex of three polypeptides of 70, 34 and 13 kD, essential for SV40 DNA replication *in vitro* (Wobbe *et al.*, 1987; Fairman *et al.*, 1988; Ishimi *et al.*, 1988; Wold *et al.*, 1988; Tsurimoto *et al.*, 1989) and also excision repair in animal cells (Coverley *et al.*, 1991). The 70 kD subunit from human cells binds to single-stranded DNA, and supports unwinding of the SV40 origin. RPA from *S. cerevisiae* is also composed of polypeptides of similar molecular weight, and the genes for each of the subunits are essential for viability (Brill *et al.*, 1989, 1991; Heyer *et al.*, 1990), indicating that

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RPA will also be essential for human chromosomal replication. In our previous experiment, the interaction with p53 prevented RPA from binding to ssDNA and, therefore, suggested a fourth mechanism by which p53 could inhibit DNA replication.

In the present study, we have mapped the RPA binding site of p53 and found that mutations in amino acids 53 and 54 (from tryptophan and phenylalanine to serine residues) in the N terminus abolish RPA binding. RPA was previously shown to bind to the transcriptional activation domains of Herpes Simplex virus transactivator VP16, of the yeast trans-activator Gal4 and the Bovine Papilloma virus trans-activator E2 (He *et al.*, 1993; Li *et al.*, 1993). Interestingly, we demonstrate a similar binding motif in the VP16 RPA binding domain. We have compared the ability of the 53,54 mutant to suppress growth in SaOs2 and H1299 cells with a mutant deficient in both transactivation and transrepression functions. The mutant unable to bind to RPA but which retained both transactivation and transrepression functions retained growth suppression activity comparable to that of wild type p53. However, a mutant defective in both transactivation and transrepression (amino acids 22,23) functions significantly lost growth suppressive activity compared to that of wild type. The loss of p53's transcription activity with mutations in 22,23 residues is known (Lin *et al.*, 1994). However, to our knowledge, this is the first report that this same mutant also significantly loses transrepression activity from a CMV promoter, a fact that should be considered when attributing biological functions of p53 solely to the loss of transactivation function by this mutant.

These studies suggest that RPA binding by p53 may be less critical in growth suppression than its ability to activate or repress transcription in SaOs2 and H1299 cells. However, the involvement of the interaction in other cellular processes, e.g. DNA repair, apoptosis, have not been studied and deserve further investigation, especially since p53-induced transcription is dispensable for many of these functions.

Results

Sub-domains of p53 synergize to give strong RPA binding

GST fusion proteins containing various fragments of p53 were generated, bound to glutathione agarose beads and their ability to bind RPA examined by affinity chromatography (Figure 1). Fragments of p53 are named by the position of the amino acids in the complete p53 sequence. We have shown earlier that two domains of p53, N2 (amino acids 2–121) and 5C (amino acids 289–393), could independently bind RPA. The domain containing amino acids 2–71 of p53 had equivalent RPA binding activity as 2–121 (data not shown). However, further dissection of sub-domains containing amino acids 2–45 or 46–71 showed much reduced RPA binding activity. Ten times as much of each GST-sub-domain protein (e.g. GST 2–45 or GST 46–71) were compared to GST-domain protein (GST 2–71) in their ability to bind RPA. The binding activity of each sub-domain was less than one-tenth that of the corresponding domains

(Figure 1b). Thus the better binding of RPA by a domain (e.g. 2–71) is probably not a simple summation of RPA binding by each of the sub-domains (e.g. 2–45 and 46–71). Similarly, at the C terminal end, the domain containing amino acids 289–356 had significant RPA binding; but sub-domains 289–330 or 331–356 did not have significant RPA binding. Here, too, there was a synergy between the two sub-domains in binding to RPA rather than a simple summation of binding by each of the sub-domains (data not shown). It is unlikely that in two separate instances the absence of RPA binding by the sub-domains is due to the RPA binding site spanning the site of division and thus being disrupted in each sub-domain. The alternative explanation is that weak RPA binding sites in each of the sub-domains synergize to produce the strong binding activity of the corresponding domain and this explanation is supported by additional data.

Aromatic amino acids in a sub-domain of p53 are important for RPA binding

The transcriptional trans-activator VP16 has been shown to interact with RPA, and a phenylalanine to proline mutation in VP16 shown to diminish RPA binding (He *et al.*, 1993; Li *et al.*, 1993). Reasoning that a similar mechanism of interaction occurred between RPA and p53, point-mutations were made in p53 which changed two adjoining aromatic amino acids, tryptophan and phenylalanine (residues 53–54) in one of the sub-domains of N2 to serines (W53S-F54S). This GST fusion protein did not bind RPA (data not shown). Several other point mutations have also been made in the N terminal part of p53 in the laboratory of Dr A Levine (Lin *et al.*, 1994), and a representative collection of these and W53S-F54S were engineered into GST-p53 fusion proteins and their RPA binding activity determined (Figure 2). The

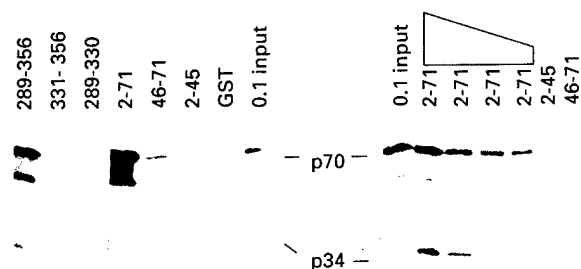


Figure 1 Sub-domains of p53 synergize to give strong RPA binding. (a) RPA bound to GST fusion proteins containing various p53 fragments (amino acids) 2–45, 46–71 and 2–71 in the N terminus; and amino acids 289–330, 331–356 and 289–356 in the C terminus) was detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. The anti-p34 antibody has a weaker titer than the anti-p70 antibody, so that at low RPA quantities, only p70 is visible. (b) Immunoblot probed with anti-p70 and anti-p34 showing titration of GST-p53 2–71, including levels of 100, 50, 30 and 10 percent the levels of both 2–45 and 46–71 sub-domains. Ten times as much of each sub-domain bound less RPA than the whole 2–71 domain. 0.1 input = one-tenth of input incubated with each of the GST fusion proteins. GST = negative control: no p53 fusion protein

results demonstrate that the aromatic residues W53 and F54 are important for RPA binding. Mutations in amino acids 48–49 (D48H-D49H) also decreased RPA binding, suggesting that negatively charged amino acids near the hydrophobic residues at 53–54 were important for RPA binding. The mutations which changed amino acids 22–23 of p53 (L22Q-W23S) affect its ability to activate transcription (Lin *et al.*, 1994), but did not affect its ability to bind RPA. Thus although in the herpesvirus transcriptional activator VP16 the same amino acid (F442) is important for both interaction with RPA and activation of transcription, this is not the case with p53. Therefore it seemed likely that we could separate the trans-activation and RPA binding functions of p53 with appropriate point-mutations.

Quantitation of RPA binding by increasing quantities of GST-p53 W53S-F54S vs GST-wild type p53 showed that the mutant was at least 10-fold weaker than wild type p53 at binding to RPA (Figure 3).

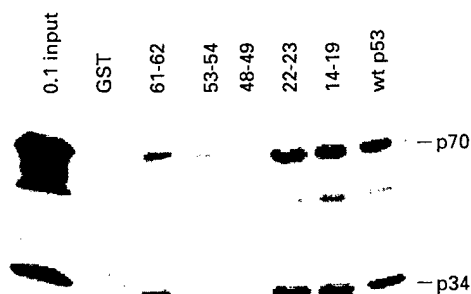


Figure 2 p53 mutants and RPA binding. RPA bound to GST fusion proteins containing p53 wild type (w.t.) and mutants were detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. The numbers above refer to the position of amino acids mutated from the original p53 wild type sequence as follows: L14Q-F19S; L22Q-W23S; D48H-D49H; W53S-F54S; and D61H-E62K. 0.1 = one-tenth S100 extract input. The GST = negative control: no p53 fusion protein

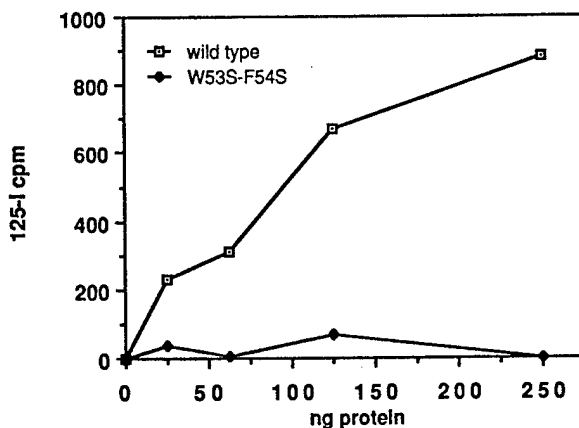


Figure 3 Titration of GST p53 wild type and W53S-F54S mutant proteins and RPA binding. Increasing amounts of GST-p53 or GST-W53S-F54S mutant proteins were added to 135 μ g S100 extract. RPA binding was detected with immunoblotting with monoclonal anti-RPA 70 and 34 subunits, followed by 125 I-labeled rabbit anti-mouse antibody. 125 I-labeled bands were excised and counted in a gamma counter

A multimeric peptide containing aromatic and charged residues binds RPA

If a weak RPA binding site has aromatic and negatively charged residues, and if synergy between weak binding sites contribute to strong RPA binding we would predict that multimerization of a putative weak binding site would create a polypeptide that binds RPA well. Multimers of a twelve amino acid peptide surrounding the W53-F54 of p53 were fused to a GST protein and their ability to bind RPA tested (Figure 4). A monomer or even a dimer of the peptide bound RPA poorly, but a trimer and higher order multimers interacted with RPA strongly. A similar multimer of a peptide but with the critical tryptophan and phenylalanine changed to serines did not bind RPA.

To examine if this was a general mechanism by which other trans-activators also bound RPA, multimers of peptides from VP16 were tested for RPA binding (Figure 5 and Table 1). A list of all the peptides which possessed RPA binding activity (Table 1) supports the general rule that aromatic amino acids surrounded by negatively charged residues contribute to RPA binding. The variation in the lengths of the peptides which can bind RPA upon multimerization suggest that most of these multimers are not in a rigid structure like an alpha helix or beta sheet because such a structure would put constraints on the lengths of the repeating units that produced a functional RPA binding polypeptide.

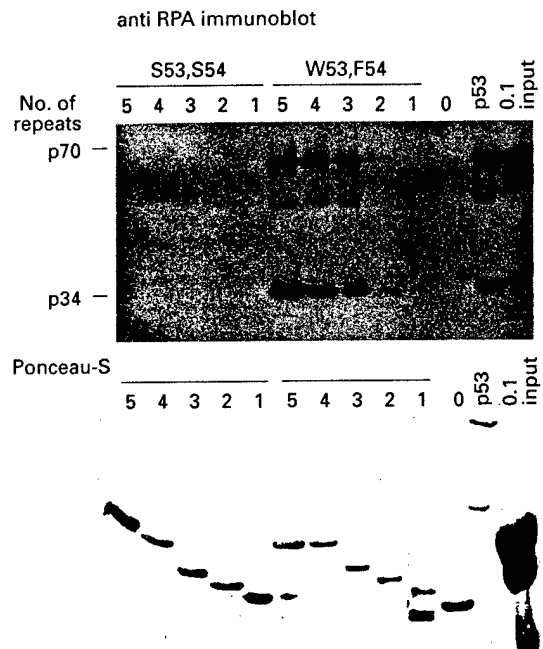


Figure 4 GST fusion proteins containing multimers of peptides from p53 wild type (W53,F54) and mutant (S53,S54) and the ability to bind to RPA. RPA bound to GST fusion proteins was detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. Ponceau S staining of the blot (bottom) demonstrates equal amounts of GST fusion protein loading in each lane. The carrier protein, casein, can also be seen in the input lane. p53 = GST p53; 0 = GST; 1, 2, 3, 4, 5 = number of repeats; 0.1 = one-tenth RPA input

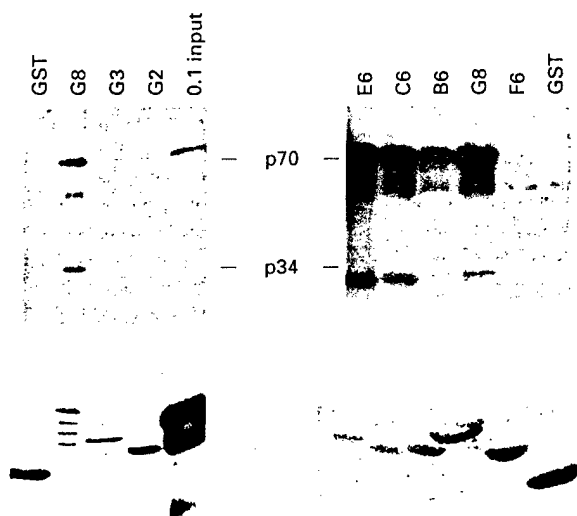


Figure 5 Ability of GST fusion proteins containing multimers of peptides derived from VP16 to bind to RPA. The sequences and amino acid position of the VP16 peptides that were multimerized (E,C,B,G,F) are in Table 1. The number after each letter indicates the number of repeats of each peptide (6 for E,C,B, and F; and 2,3, and 8 for G). RPA bound to these GST fusion proteins was detected as noted in Figure 1. Ponceau S staining of the blot (bottom) demonstrates equal amounts of GST fusion protein loading in each lane. GST=negative control; 0.1 input=one-tenth RPA input

Table 1 p53 and VP16 peptides binding to RPA

	Peptide	RPA binding
p53:	48 58 WF: DDIEQWFTEDG	+
	48 58 SS: DDIEQSSTEDG	-
VP16:	469 476 E: DMADFEFE	+
	437 445 B: DALDDFDLD	+
	437 448 G: DALDDFDLDMLG	+
	441 448 C: DFDLDMLG	+
	422 428 F: DELHLDG	-

The peptide sequence shown is the monomer that has been multimerized for RPA binding experiments (see methods). The sequences are derived from VP16 (E, B, G, C, F) and p53 wild type (WF) and mutant (SS). The numbers above each indicate amino acid number in the original protein. F is common in all the peptides that bind RPA

RPA from crude cell extracts does not bind to the 5C domain of p53

The W53S-F54S mutation in p53 produced a significant decrease in the binding of RPA from crude cell extracts (S100 from 293 cells) (Figure 2), even though the C terminal 5C domain of p53 had also been shown to interact with purified RPA (Dutta *et al.*, 1993). One explanation could be that 5C is unable to bind RPA from cell extracts. When tested directly, we found that while N2 could bind RPA from both

purified fractions and from cell extracts, 5C could only bind RPA from the former (Figure 6). This effect was confirmed in cell extracts from SaOs2, H1299 and WRL68 (human embryo liver) cells. The above observation explains how we obtained a mutant form of p53 which loses the ability to bind RPA from cell extracts by making mutations only in the N2 (amino acids 2-121) domain of p53, leaving intact the dimerization and nuclear localization functions in the 5C domain which are essential for growth suppression.

Transcription activation by p53 mutants

To test the transcription activation properties of these p53 molecules, a transient transfection assay was done (Figure 7). Plasmids expressing p53 and various mutant derivatives were co-transfected into SaOs2 and H1299 cells (which lack endogenous p53) with a reporter plasmid, 6FSVCAT, which has six consensus p53 binding sites cloned upstream from a CAT gene (Unger *et al.*, 1993). Transcription activation by p53 was lowest in the L22Q-W23S mutant of p53 (approximately 25% of wild type), although it still retained fivefold (in SaOs2) and threefold (in H1299) activation over vector control. A defect in the transcription activation function of this mutant was first reported by Lin *et al.* (1994) where activity was comparable to that of vector alone. L14Q-F19S, D48H-D49H and D61H-E62K mutant forms of p53 retained 45-70% of transcriptional activity, comparable to activities also reported by Lin *et al.* (1994). The D48H-D49H and W53S-F54S versions of p53 possessed at least 45% wild-type trans-activation levels but significantly diminished RPA binding activities. Transcription activity of these mutants was confirmed by co-transfecting H1299 cells with another reporter plasmid, cosX1CAT, which contains the p53-responsive promoter of the mdm2 gene directing the expression of CAT (Lin *et al.*, 1994).

Plasmids expressing p53 mutants were transiently transfected into SaOs2 and H1299 cells, and cell lysates immunoblotted by Western analysis with anti-p53 antibody (1801, Oncogene) to ensure that protein expression of p53 mutants was comparable to that of

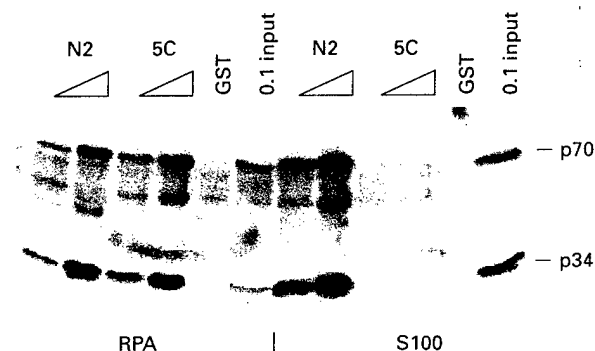


Figure 6 p53 5-C does not bind to RPA in crude cell lysates. RPA bound to GST fusion proteins p53 N-2 (amino acids 2-121) and p53 5-C (amino acids 289-393) were detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. GST=negative control; 0.1=one-tenth input, which was pure RPA (left) and 293 cell extracts (S100) (right)

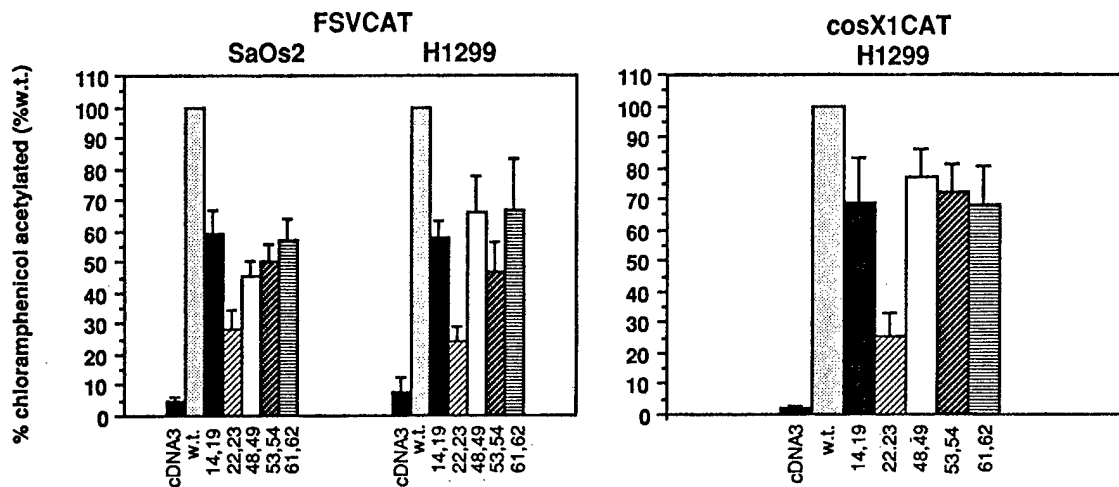


Figure 7 6FSV-CAT activity of p53 wild type and mutants transiently transfected into SaOs2 and H1299 cells (left) and cosX1-CAT activity in H1299 cells (right). CAT activity represents percent 14 C-chloramphenicol acetylated compared to p53 wild type (=100% activity) and represents the mean \pm SEM activity of eight plates from each mutant

wild type (data not shown). The same result was obtained by estimating the levels of p53 expression by immunofluorescence with anti-p53 antibodies. Because the D48H-D49H epitope was not recognized by the monoclonal antibody 1801, expression of this mutant was determined with the DO-1 antibody (Santa Cruz Biotechnology).

Transcription repression by p53 mutants

p53 represses transcription from TATA box containing promoters that do not have p53 binding sites, and the region of p53 responsible for this activity mapped to approximately the same region responsible for transcription activation (Lin *et al.*, 1994). Transcription from the immediate early promoter of cytomegalovirus is

repressed by p53 (Crook *et al.*, 1994; Subler *et al.*, 1994). We used the battery of mutant p53 expressing plasmids to determine how the mutations affect transcription repression (Figure 8). L14Q-F19S, D48H-D49H, W53S-F54S and D61H-E62K mutants of p53, which retained most of the trans-activation function, retained at least 50% of their trans-repression function. The L14Q-F19S mutant retained 40% of this function in H1299 and SaOs2 cells. L22Q-W23S, the mutant which was most reduced in transcription activation, was also the most impaired in transcription repression and was comparable to leaving out the plasmid expressing p53 (cDNA3 alone = 100% loss of repression). This result suggests that the same residues of p53 involved in trans-activation and in contacting the basal transcription apparatus are also important for trans-repression.

Growth suppression by p53 mutants

Plasmids expressing wild type or mutant p53 were transfected into SaOs2 and H1299 cells (deficient in endogenous p53) and G418 resistant colonies selected (Figure 9). As demonstrated by others, plasmids expressing wild type p53 established very few G418 resistant colonies compared to the vector which does not express p53, due to growth suppression by p53. The results from the other plasmids demonstrate that both p53 proteins with wild type transcription trans-activation but diminished RPA binding, D48H-D49H and W53S-F54S, exhibited as much growth suppression as wild type p53 proteins. Therefore, both forms of p53 with diminished RPA binding retained growth suppression.

The p53 protein L22Q-W23S, which had wild type RPA binding activity, reduced transcription trans-activation, as well as transcription repression, showed diminished growth suppression in both SaOs2 and H1299 cells. The L14Q-F19S and D61H-E62K mutants, which retained most of the trans-activation and repression functions, also retained most of the growth suppression activity of wild type p53 in both

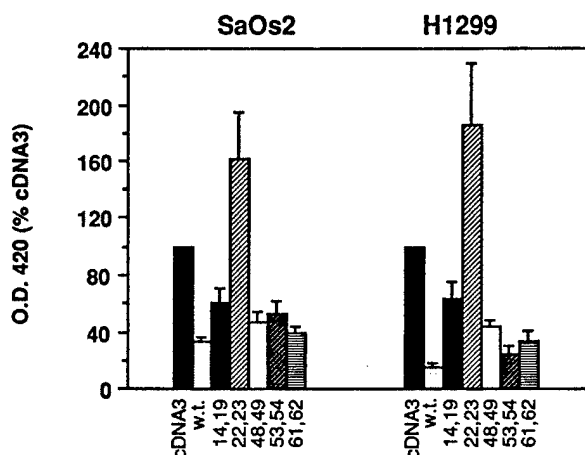


Figure 8 Beta-galactosidase activity of p53 wild type and mutants transiently transfected into SaOs2 and H1299 cells. Beta-galactosidase activity represents the O.D. 420 after addition of ONPG substrate compared to vector cDNA3 alone (=100%, no transcription repression) and represents the mean \pm SEM activity of eight plates from each mutant

SaOs2 and H1299 cells. These results imply the trans-activation and/or repression properties of p53 are important for growth suppression.

To ensure that we did not miss a subtle effect of the W53S-F54S mutation on the ability of p53 to suppress entry into S phase, a transient transfection assay was done. Plasmids expressing p53 proteins were introduced into SaOs2 or H1299 cells and entry into S phase measured by BrdU incorporation as described by Delsal *et al.* (1995). BrdU was fluorescently detected by phycoerythrin-conjugated anti-mouse IgG2a (subclass of anti-BrdU primary antibody). p53 expression was detected by FITC-conjugated rabbit anti-mouse IgG1 (subclass of anti-p53 (1801) primary antibody). We found that the W53S-F54S mutant had slightly decreased ability to stop entry of cells into S phase. However, several other mutants of p53 (which retain RPA binding) were equally diminished (data not shown). Therefore, we believe that the RPA binding activity of p53 is not critical for growth suppression.

Discussion

Relatively short peptides with aromatic amino acids surrounded by negatively charged residues produce RPA binding activity when repeated several times in a

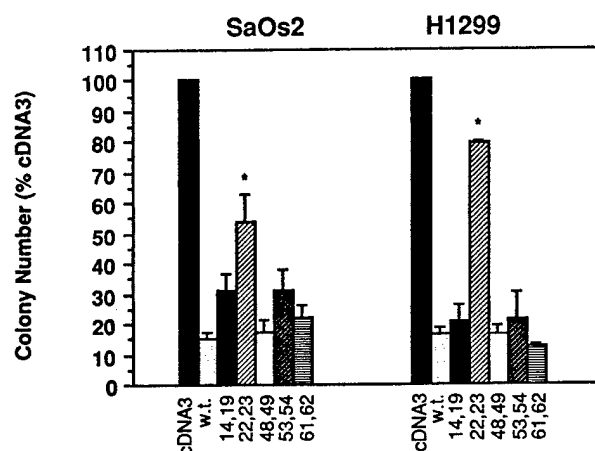


Figure 9 Growth suppression of wild type p53 and mutants in stable transfection assays. Bars represent the mean \pm SEM of the number of colonies for 14 (SaOs2) and 4–5 transfections (H1299) compared to cDNA3 (=100%, no growth suppression). Data were analysed by one-way ANOVA and means were categorized by Fisher's LSD test. *indicates a significant difference compared to all other p53 alleles at $P < 0.0003$ (SaOs2) and $P < 0.0001$ (H1299)

Table 2 Summary of results

p53	RPA binding	Transcription activation	Transcription repression	Growth suppression
wild type	+	+	+	+
L14Q-F19S	+	+	+	+
L22Q-W23S	+	–	–	–
D48H-D49H	–	+	+	+
W53S-F54S	–	+	+	+
D61H-E62K	+	+	+	+

protein. The degenerate nature of this RPA binding signal probably accounts for a number of proteins reported to bind RPA. Since similar bulky hydrophobic residues in the context of negatively charged amino acids are also essential for transcriptional trans-activation (Cress *et al.*, 1991), this observation may explain why several transcriptional activators are able to bind RPA. Potentially, this could be a common feature of several protein-protein interactions. The recently reported interaction between the DNA repair protein XP-G and RPA also uses a negatively charged part of XP-G (He *et al.*, 1995), and it is possible that the same mode of interaction is involved. The creation of a potent RPA binding site by multimerization of a slightly degenerate peptide module is novel. We suspect that several biologically relevant low affinity interactions (such as those cited above and those between transcriptional activators and the basal transcription apparatus) could be mediated by similar interaction surfaces.

Bulky hydrophobic residues in the N terminal region of p53 have already been implicated in transcriptional trans-activation and in interactions with the TATA binding protein and viral and cellular oncogenes E1b and mdm2 respectively (Lin *et al.*, 1994). Although our results suggest that at least for RPA binding, the interaction depends on relatively degenerate sequences, there must be some specificity to the modules that interact with transcriptional factors vs the ones that interact with RPA because we were able to create point mutations which affected RPA binding but not trans-activation and vice-versa. The exact source of this specificity will become more clear when the interaction domains of the other partners (RPA, TAFII40, E1b or mdm2) in these interactions are defined.

Peptides of varying lengths could be multimerized to produce RPA binding. Therefore, there do not appear to be strict structural constraints on the interactor modules, because each of the multimers have different distances between the aromatic residues, between the acidic residues and between the aromatic and acidic residues. We think that the interactor modules are unstructured, and may be induced into a more defined structure when the other partner is bound. Recent biophysical assays indeed demonstrated that the transcriptional activation domain of VP16 is relatively unstructured in the free state and acquires some structure when complexed to the basal transcriptional apparatus (Shen *et al.*, 1996 a,b). This 'induced fit' hypothesis also leaves room for specificity of interaction depending on the structure of the other partner in the interaction. The minimal requirements of the other partner could be to have a distribution of bulky hydrophobic residues surrounded by positively charged residues, so that hydrophobic and electrostatic interactions would stabilize the interaction.

We do not yet know why the 5C region did not bind to RPA from the crude cell extracts. Either the RPA from cell extracts is present in a form where it is not able to interact with 5C, or the cell extract contains factors which bind to 5C and prevent RPA from binding. The effect is probably not due to pre-existing p53-RPA complexes, since two cell lines in which the effect was shown, SaOs2 and H1299, lack functional alleles of p53. The TATA box binding protein (TBP) binds to the 5C region (Horikoshi *et al.*, 1995).

Potentially, TBP or similar factor(s) could bind to 5C in cell extracts and prevent RPA from binding to the same.

L22Q-W23S showed decreased transcription activation, loss of transcription repression, wild type RPA binding and decreased growth suppression, indicating that transcriptional trans-activation and/or transcription repression is most important for growth suppression. RPA binding, in contrast, lost in the D48H-D49H and W53S-F54S alleles of p53, appears unimportant for growth suppression.

As mentioned in the introduction, p53 has other functions relevant to the production of cancers. It is required to induce apoptosis in response to x-irradiation or chemotherapy, to produce a pause in DNA replication after a sub-lethal dose of radiation so as to give the cell time to repair its DNA, and to prevent gene amplification. p53 can induce apoptosis through a pathway independent of new mRNA transcription and protein synthesis (Caelles *et al.*, 1994), making it likely that the transcriptional trans-activation function of p53 is occasionally dispensable for this activity. p53 has recently been shown to selectively bind to insertion-deletion mismatch lesions (Lee *et al.*, 1995), and by analogy with the XP-A-RPA interaction (He *et al.*, 1995), may be involved in recruiting RPA to these sites of DNA repair. The p53-RPA interaction could be important for apoptosis induction and/or the other functions of p53 described in this paragraph. Future experiments will be directed toward testing these possibilities.

An examination of which p53 mutants retain or lose trans-repression suggests possible mechanisms for the trans-repression. Hydrophobic residues at 22–23 important for trans-activation are also required for trans-repression so that sequestration of basal transcription factors by p53 is likely to be important for trans-repression. The sequestration could be executed through the low specificity interactor modules containing hydrophobic residues as described for the RPA-p53 interaction. Data from other groups has suggested that the oligomerization domain at the C terminus of p53 is important for trans-repression activity. (Subler *et al.*, 1994). Therefore trans-repression probably involves multimers of p53 sequestering basal transcription factors in complexes away from functional promoters.

In conclusion we have determined which feature of p53 and other trans-activators promotes interaction with RPA, and shown with two mutant alleles of p53 that growth suppression occurs independent of binding to RPA. We have also shown that trans-repression by p53 is affected by mutations at residues 22–23 (which are also important for trans-activation), and this allele is most defective in growth suppression. These results suggest that while RPA-p53 interaction is not important, transcription trans-activation and/or trans-repression by p53 are important for cell growth suppression.

Materials and methods

p53 wild type and mutant constructs

p53 fragments (amino acids 2–71; 2–45; 46–71; 1–121; 289–393; 289–330; 331–356) were generated by PCR with appropriate pairs of oligonucleotides as primers using a

clone of p53 cDNA as a template. Fragments were then cloned into BamHI and Asp 718 sites of pet11GTK vector (Dutta *et al.*, 1993), such that the GST protein reading frame was fused in frame with the p53 fragment. To make the W53S-F54S allele of p53, site-directed mutagenesis was performed by both a PCR based method and the Kunkel method (Sambrook *et al.*, 1989). Mutant alleles were sequenced to ensure that no additional mutations were created.

Peptide multimers

Oligonucleotides corresponding to p53 amino acids 48–58 (both wild type and W53S, F54S mutants) were synthesized. Partial BglII and BamHI sites were generated at the ends of the oligos to facilitate the oligomerization. Corresponding oligonucleotides were phosphorylated, annealed and ligated to BamHI site of pet11GTK. Clones containing insertions were screened by colony PCR. All clones containing different numbers of insertions were rechecked by PCR and confirmed by sequencing. The GST fusion proteins containing multimers of peptides from VP16 will be described elsewhere (M Tanaka, unpublished observations).

RPA-p53 interaction assay

The GST-p53 fusion proteins were produced and purified on glutathione agarose beads as described previously (Dutta *et al.*, 1993). Beads carrying 400–600 ng GST and 200–300 ng GST p53 fusion proteins were used in the assays, and incubated with either 125 ng pure RPA or 135 μ g S100 extract from 293 cells (transformed primary embryonal kidney, human) as indicated. RPA purification from human 293 cell extracts, and the assay for binding of RPA by GST-p53 fusion proteins has also been described (Dutta *et al.*, 1993). For the accurate quantification of RPA binding shown in Figure 3, the Westerns were developed with ¹²⁵I labeled rabbit anti-mouse IgG (Dupont Chemicals). ¹²⁵I labeled bands were excised and counted in a gamma counter.

Growth suppression by stable transfections

CMV/p53 mutants 14–19, 22–23, 48–49 and 61–62 were the kind gift of Dr Arnold Levine. p53 wild type and W53S-F54S mutants were cloned into a mammalian expression vector cDNA3 (Invitrogen) which expresses genes inserted downstream from a cytomegalovirus (CMV) promoter and which contains a neomycin phosphotransferase gene and an SV40 origin of DNA replication. These plasmids were transfected into SaOs2, a human osteosarcoma cell line with loss of both alleles of p53, as well as H1299, a human lung large cell carcinoma cell line with partial homozygous deletion of the p53 gene, by the calcium phosphate method. Exponentially growing cultures were transfected with 10 μ g of each plasmid. After 24 h, cells were washed in phosphate buffered saline and fresh DMEM medium containing 10% fetal calf serum and G418 was added. The ability of each plasmid to produce G418 resistant colonies was measured as described (Chen *et al.*, 1995).

Transcription activation and repression

SaOs2 or H1299 cells were transfected with 10 μ g of plasmids expressing p53 alleles (based on the cDNA3 vector), 5 μ g of a reporter plasmid, 6FSVCAT, expressing the chloramphenicol acetyl transferase (CAT) gene downstream from a p53 consensus binding sequence containing six copies of the p53-binding element TGCCT (Unger *et al.*, 1993). Transcription activity was confirmed in H1299

cells by transfecting as above with a cosX1CAT plasmid containing a p53-responsive promoter from murine mdm-2 gene (Wu *et al.*, 1993). Activity in SaOs2 cells was not tested with this construct due to a high level of background CAT activity. Transcription repression was tested by transfecting as above with a reporter plasmid expressing the beta-galactosidase gene from a cytomegalovirus promoter known to be repressed by wild type p53 (Crook *et al.*, 1994; Subler *et al.*, 1994). Eight hours later, plates were washed twice in PBS and fresh medium (DMEM with 10% FCS) was added. After 36 h cells were harvested and lysed. Equal fractions of cell lysates from each of the transfected plates were assayed for CAT (transcription activation) and beta-galactosidase (transcription repression) activity (Sambrook *et al.*, 1989). CAT and beta-galactosidase activity were expressed as percentage of activity relative to plates with wild type (100%) and cDNA3 vector alone (100%), respectively.

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Statistics

Data for growth suppression assays were statistically analysed by one-way analysis of variance, and means were categorized by Fisher's LSD test (Steel *et al.*, 1980).

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Cyclin-Binding Motifs Are Essential for the Function of p21^{CIP1}

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The cyclin-dependent kinase (Cdk) inhibitor p21 is induced by the tumor suppressor p53 and is required for the G₁-S block in cells with DNA damage. We report that there are two copies of a cyclin-binding motif in p21, Cy1 and Cy2, which interact with the cyclins independently of Cdk2. The cyclin-binding motifs of p21 are required for optimum inhibition of cyclin-Cdk kinases *in vitro* and for growth suppression *in vivo*. Peptides containing only the Cy1 or Cy2 motif partially inhibit cyclin-Cdk kinase activity *in vitro* and DNA replication in *Xenopus* egg extracts. A monoclonal antibody which recognizes the Cy1 site of p21 specifically disrupts the association of p21 with cyclin E-Cdk2 and with cyclin D1-Cdk4 in cell extracts. Taken together, these observations suggest that the cyclin-binding motif of p21 is important for kinase inhibition and for formation of p21-cyclin-Cdk complexes in the cell. Finally, we show that the cyclin-Cdk complex is partially active if associated with only the cyclin-binding motif of p21, providing an explanation for how p21 is found associated with active cyclin-Cdk complexes *in vivo*. The Cy sequences may be general motifs used by Cdk inhibitors or substrates to interact with the cyclin in a cyclin-Cdk complex.

The periodic activation and inactivation of cyclin-dependent kinases (Cdks) is essential for the progression of a cell through each phase of the cell cycle (29, 39). The activity of a given Cdk is regulated by synthesis of the corresponding cyclin in specific stages of the cycle and by posttranslational modifications on the Cdk subunit. Inactivation of the kinase is regulated by destruction of the cyclin, by posttranslational modifications on the Cdk subunit, and by newly discovered inhibitors which associate with the cyclin-Cdk. There are two such related families of inhibitors. The first family includes p21 (7, 10, 14, 32, 45), p27 (16, 35, 36, 43), and p57 (23, 28) and acts on a wide range of Cdks. The second family of inhibitors consists of p15, p16, and p18 (11, 13, 37) and specifically inhibits the cyclin D-dependent G₁ kinases Cdk4 and Cdk6.

Interest in the Cdk inhibitors has been high because of strong evidence of their involvement in the regulation of cell growth and differentiation. p21 is transcriptionally induced by the tumor suppressor protein p53 (7). The transforming mutations of p53 commonly seen in human cancers fail to induce p21 in response to DNA damage. Embryonic fibroblasts from mice lacking functional p21 fail to stop at the G₁-S transition following radiation-induced DNA damage (1, 4). Since this block is controlled by wild-type p53, p21 probably is the primary effector of this particular action of p53 (6). In addition, p21 may be required to stop cell proliferation prior to differentiation (12, 34, 41). Although p21 knockout mice retain normal tissue differentiation, the existence of closely related p21 family members such as p27 and p57 suggests that p21 may be functionally redundant. The p15/p16 class of Cdk inhibitors has been implicated more directly in the regulation of cell growth (19, 31). Several tumors and tumor-derived cell lines have deletions in the p15 and p16 genes, and at least one form

of familial predisposition to melanomas is attributed to germ line mutations of these genes.

Most of the biological activities of the p21/p27/p57 and p16/p15/p18 families depend on their ability to inhibit cyclin-Cdk kinases. In addition, p21 also associates with and inhibits the DNA replication and repair factor proliferating cell nuclear antigen (PCNA), which could be important to stall active DNA replication forks following DNA damage (3, 8, 25, 27, 33, 40, 42, 44). However, the block to the G₁-S transition is executed by mutant forms of p21, which inhibit Cdk but not PCNA (3, 27). *In vitro*, the onset of DNA replication in *Xenopus* egg extracts is inhibited primarily by the Cdk inhibition activity of p21 (3). These studies suggest that the kinase-inhibitory activity of p21 is responsible for its growth suppression function *in vivo*. The biological roles of the p21-PCNA interaction and of the p21-cyclin-Cdk-PCNA complex are still unclear.

Both families of Cdk inhibitors require stable association with the Cdk subunit for kinase inhibition. Mutations in p21 which affect its association with the Cdk subunit decrease its ability to inhibit the kinase (3, 9, 27, 30). Likewise, mutations in p16 which disrupt association with Cdk4 abrogate its kinase-inhibitory activity (20). However, the p21 family associates with the cyclin-Cdk complex without displacing the cyclin, while p16 competes with the cyclin D for association with Cdk4 or Cdk6. The mechanism by which p21 inhibits the cyclin-Cdk kinases is still unknown.

In this report, we demonstrate that p21 directly associates with cyclins. This association is mediated through a conserved region near the N terminus of p21, which we refer to as cyclin-binding motif 1 (Cy1). Cyclin D1 also associates with p21 through the Cy1 sequence motif but only when complexed with the Cdk4 subunit. A second, weaker cyclin-binding motif, Cy2, was discovered in the C-terminal region of p21. Deletion mutants of p21, synthetic peptides containing the Cy1 and Cy2 motifs, and a monoclonal antibody which recognizes an epitope overlapping Cy1 were used to demonstrate the importance of the cyclin-p21 interaction for kinase inhibition and for biological activity of p21. The cyclin-binding motif is present in

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other molecules reported to interact with cyclin-Cdk complexes, suggesting that those interactions are also mediated via the cyclin subunit of the cyclin-Cdk complex.

MATERIALS AND METHODS

Expression plasmids and baculoviruses. Plasmids used for expression of various proteins in bacteria were pETp21His (44), pETp27His (36), pETCdk2 (3), pGST-RbC (20), pGST-cyclin D1,2,3 (from Yue Xiong), and pGST-cyclin E (17). A *Bam*HI-*Hind*III fragment of cyclin E from pGST-cyclin E was cloned into the *Escherichia coli* expression vector pRSET (Invitrogen) to generate pRSET-cyclin E (26-402). pGST-cyclin E (1-127) was generated by removing the *Pst*I-*Hind*III fragment from pGST-cyclin E. Similarly, pGST-cyclin E (1-216), (1-326), and (1-334) were generated by removing *Nco*I-*Hind*III, *Nco*I (second)-*Hind*III, and *Sac*I-*Hind*III fragments, respectively.

Plasmids containing the mutant alleles of p21 were obtained from J. Smith (30). PCR with N- and C-terminal oligonucleotides was used to clone these mutant alleles of p21 into pGEX-5X-3. pGEX-p21 Δ 1-29 was made by restriction enzyme digestion of a plasmid expressing glutathione S-transferase (GST)-p21, using a *Pvu*II site in the coding region of p21. pGEXp21 Δ 17-24 was made by a PCR-based strategy using appropriately designed oligonucleotides. Fragments containing p21 coding regions with deletions at either amino acids 17 to 24 or amino acids 53 to 58 also were cloned into pETHis (36) for expression of His-tagged p21 derivatives in bacteria.

pCMVCdk2, pCMVCdk4, pRcCyclin A, pRcCyclin B, pRcCyclin E, and pRcCyclin D1, D2, and D3 were kindly provided by P. Hinds and used for the expression of cyclins and Cdks by *in vitro* transcription and translation.

Baculoviruses expressing Cdk2, GST-cyclin A, GST-cyclin B, and GST-cyclin E were obtained from Helen Piwnicka-Worms. Baculoviruses expressing cyclins D1, D2, and D3 were gifts from C. J. Sherr. Baculovirus expressing GST-Cdk4 was provided by J. W. Harper (see reference 15 and references therein).

Mammalian cell expression constructs containing full-length p21 and p21N have been described previously (3). p21 Δ 17-24 and p21N Δ 17-24 were cloned into pDNA3 (Invitrogen) by using the same strategy.

Protein expression and purification. Bacterial expression of proteins was performed in *E. coli* BL21. Protein induction, cell lysis, and affinity purification with glutathione-agarose beads (Sigma) were done as described previously (5).

Hi-5 cells were infected with recombinant baculoviruses containing various cyclins and Cdks as described previously (15). Active cyclin-Cdk complexes were affinity purified with glutathione-agarose beads (Sigma).

Protein expression by *in vitro* transcription and translation was performed by using the TnT coupled rabbit reticulocyte lysate system (Promega).

Peptides PS100 (ACRRFLFGPVDSE), PS101 (ACRRLLKPPVDSE), PS102 (FYHSKRRLIFSK), and PS103 (FYHSKRDDIFSK) were synthesized by Research Genetics Inc. A 41-amino-acid p21C2 peptide (QAEGSPGGPGDSQGR KRRQTSMTDFYHSKRRLIFSKRPKK, consisting of the 39 C-terminal amino acids of p21 and two lysine residues at the carboxy-terminal end required for chemical synthesis) was synthesized at the Harvard Medical School Biopolymer Laboratory. BWH262 (WNSGFESYGSSSYGGAGGYTQAPGGFGAPAPSQ AEKKSRRAR) was used as a control peptide from the N terminus of human RPA p34.

Pull-down assay, immunoprecipitation, and immunoblotting. Pull-down assays were performed as described previously (3). Basically, 100 to 300 ng of GST fusion protein and 5 to 10 μ l of bacterial or reticulocyte lysate in 200 μ l of buffer A7.4 (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 25 mM NaCl) were incubated for 1 h at 4°C on a rotating wheel. Proteins associated with GST fusion proteins were pulled down with glutathione-agarose beads. After the beads were washed four times in binding buffer, bound proteins were eluted by boiling in Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 10 min, separated by SDS-PAGE, and detected either by autoradiography (for radiolabeled proteins expressed in *in vitro* transcription and translation reactions using rabbit reticulocyte lysate) or by Western blotting (immunoblotting) using appropriate antibodies. In each experiment, care was taken to equalize the amounts of input proteins and GST protein was included as a negative control. For peptide competition, the indicated peptides were included in the reactions at a concentration of 75 μ M. Conditions used for the immunoprecipitation-immunoblot assay with human cell lysates were as described previously (47).

The antibodies used in this study were monoclonal antibodies to cyclin E (HE12 and HE172) and polyclonal antibodies to p27 (J. Massagué), Cdk2 (Santa Cruz), Cdk4 (H. Chou), cyclin A (J. Pines), and cyclin D1 (L. Zuckerman). The monoclonal antibodies (CP2, CP36, and CP68) to p21 were generated against full-length recombinant human p21 (47). CP2, CP36, and CP68 recognize amino acids 1 to 17, 17 to 24, and 130 to 150, respectively, of p21, as determined by immunoblotting of a panel of bacterially produced deletion derivatives of p21.

Kinase assay and *Xenopus* DNA replication assay. Kinase assays were performed for 15 min at 30°C, using 1 ng of insect cell-expressed cyclin-Cdk complexes and 3 μ g of purified GST-RbC (C-terminal portion of the retinoblastoma protein [Rb]) in 25 μ l of kinase buffer (50 mM Tris-HCl [pH 7.4], 10 mM MgCl₂, 1 mM dithiothreitol, 50 μ M ATP containing 5 μ Ci of [γ -³²P]ATP) with other indicated components. Kinase reactions were stopped by the addition of 2X

Laemmli sample buffer and then boiled for 10 min. The products were analyzed by SDS-PAGE. Phosphorylation of the substrate was quantitated both by liquid scintillation counting of bands excised from gels and by PhosphorImager analysis. K_{iapp} is the concentration of inhibitor at which kinase activity is inhibited by 50%.

For immunoprecipitation-kinase assays shown in Fig. 9, 10 ng of GST-cyclin E-Cdk2 was incubated with 1 μ g of bacterially produced p21 derivatives (or peptides) at 4°C for 30 min in 200 μ l of buffer A7.4. p21-associated proteins were immunoprecipitated with monoclonal antibody CP68, and kinase reactions were performed with GST-RbC as the substrate.

DNA replication in *Xenopus* egg extracts was carried out as described previously (21).

Growth suppression assay. The stable transfection assay for measuring growth suppression by p21 and its alleles was performed as described previously (3) except that two different cell lines, SaOs2 (human osteosarcoma) and H1299 (human lung adenocarcinoma), were used.

RESULTS

Cyclin E associates stably with p21 or p27 independently of the catalytic Cdk2 subunit. Bacterially expressed p21 or p27 was mixed with glutathione-agarose beads containing equal amounts of GST-cyclin fusion proteins to test the direct association of p21 or p27 with various cyclins (Fig. 1A, 1B, and 2A). GST-cyclin E associated best with p21 and p27, although weak interaction could be seen with GST-cyclin A and GST-cyclin B (Fig. 1B). Cyclin A-p21 association is best seen in Fig. 2A, where the amount of p21 input is 10 times more than in Fig. 1A.

Cyclin E and p21 associated with each other even when both GST-p21 and cyclin E were produced in bacteria (Fig. 1C, lane 3). The same was true for cyclin E and p27 (data not shown). Various deletion derivatives of p21 were generated to define which portion of p21 was responsible for the interaction with cyclin E. p21 with a deletion of amino acids 17 to 24 failed to bind cyclin E (Fig. 1C, lanes 4 and 5). The association of cyclin A with p21 was also disrupted by the deletion of amino acids 17 to 24 (Fig. 2A, middle panel). Thus, amino acids 17 to 24 of p21, ACRRFLFGP, form part of the cyclin-binding motif of p21 and will be referred to as the Cy1 site. This sequence is highly conserved (7-of-8-bp identity) among the Cdk inhibitors p21, p27, and p57.

The same deletion derivatives of p21 were used to bind bacterially expressed Cdk2 (Fig. 1D). Amino acids 53 to 58 of p21 were essential for association with Cdk2 (lane 8), consistent with published studies showing that this region is required for association with and inhibition of cyclin-Cdk kinases (9, 30). We refer to this Cdk-binding motif of p21 as the K site. Therefore, the cyclin-binding Cy1 site is distinct from the Cdk2-binding K site.

The cyclin box (amino acids 131 to 219) and C-terminal portions of cyclin E are essential for association with Cdk2 (24, 38). When bacterially expressed GST-cyclin E constructs containing progressively larger deletions from the C terminus of the cyclin were tested, amino acids 1 to 127 of cyclin E were found to be sufficient to bind p21 (Fig. 1E). This region is N terminal to the cyclin box and is incapable of binding Cdk2, again confirming that the direct association of p21 and cyclin E is independent of Cdk2. Identical results were seen with cyclin E and p27 (data not shown).

Taken together, these results indicate that cyclin E or cyclin A can stably associate with p21 or p27 independently of Cdk2. The association of p21 or p27 with cyclin E appears stronger than that with cyclin A.

The Cy1 site of p21 is important for interaction with cyclin-Cdk complexes. We next tested whether the Cy1 site of p21 was important for the interaction of p21 with cyclin-Cdk complexes (results summarized in Table 1). Cdk2, Cdk4, cyclin E, cyclin A, and cyclin D1 were produced separately in rabbit reticulo-

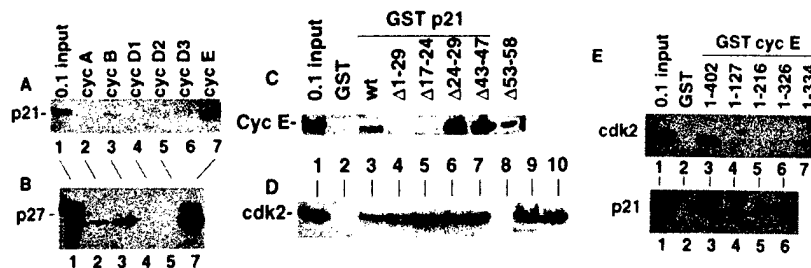


FIG. 1. Direct interaction between p21 and p27 with cyclins. (A) p21 associates directly with GST-cyclin (cyc) E. Proteins bound to indicated GST fusion proteins after incubation with an *E. coli* lysate containing recombinant human p21 were visualized by immunoblotting with polyclonal anti-p21 antibody. 0.1 input, 1/10 input lysate. (B) p27 associates directly with some GST-cyclins, cyclin E in particular. The lane numbers match those in panel A except that the input lysate was *E. coli* lysate containing His₆-tagged p27, and the protein was visualized with a polyclonal antibody to p27. (C) Amino acids 17 to 24 of p21 are essential for association with cyclin E. Protein bound to GST or GST-p21 (indicated alleles) after incubation with *E. coli* lysate containing recombinant human cyclin E was visualized by immunoblotting with monoclonal antibody HE12 to cyclin E. The 55-kDa cyclin E band is indicated. wt, wild-type p21; Δ 1-29, etc., p21 with amino acids 1 to 29, etc., deleted. (D) Amino acids 53 to 58 of p21 are essential for association with Cdk2, and loss of the cyclin-binding site (amino acids 17 to 24) does not affect binding to Cdk2. The lane numbers match those in panel C except that the input lysate was *E. coli* lysate containing recombinant human Cdk2, and the protein was visualized by a commercial antipeptide antibody to Cdk2. Lanes 9 and 10 contain protein bound by GST-p21N and GST-p21N Δ 17-24. (E) Binding of C-terminal deletions of cyclin E to Cdk2 and to p21. p27 behaves in the same way as p21 (data not shown). (Top) Bacterial lysate containing recombinant Cdk2 was incubated with agarose beads coated with GST or GST-cyclin E (indicated alleles). Cdk2 bound to the various GST derivatives was visualized by immunoblotting. Cyclin E 1-402, wild-type cyclin E; 1-127, etc., N-terminal fragment containing amino acids 1 to 127, etc., of cyclin E. (Bottom) Lane numbers correspond to those in the top panel except that the input bacterial lysate contains recombinant p21.

cyte lysates by in vitro transcription-translation (Fig. 3). When cyclin E or A was synthesized alone, immunoprecipitation with anti-Cdk2 antibody demonstrated that the radiolabeled cyclin was associated with rabbit Cdk2 from the reticulocyte lysate (Fig. 3A, lane 2, and data not shown). Likewise, when cyclin D1 was produced in the rabbit lysate, it was complexed with rabbit Cdk4 and Cdk2 (Fig. 3B, lanes 8 and 2). Cdk2 and Cdk4 produced in rabbit reticulocyte lysates were free of cyclins, as none of the antibodies against cyclins immunoprecipitated the Cdk subunits (data not shown).

The radiolabeled proteins were tested for association with GST-p21 and various deletion derivatives of p21. Cdk2 interacted exclusively with amino acids 53 to 58 of p21 (K site), because deletion of this sequence disrupted the association of Cdk2 with p21 (Fig. 3C, lane 6). Cdk4 alone did not associate with p21 (Fig. 3D, lane 3). Cyclin D1-Cdk4 complex associated with p21 exclusively via the Cy1 site because deletion of this site abolished all association (Fig. 3B, lanes 4 and 5; the residual cyclin D1 seen in these lanes is probably due to cyclin D1-Cdk2). Unlike Cdk2 alone or cyclin E alone, the association of cyclin E-Cdk2 with p21 was not disrupted by mutation of either the K or the Cy1 site (Fig. 3A, lanes 4 to 6), suggesting that both of these sites independently interact with the cyclin E-Cdk2 complex. Cyclin A-Cdk2 behaved like cyclin E-Cdk2 in these assays (Table 1).

To confirm these results, GST-cyclin E, GST-cyclin E-Cdk2, GST-cyclin A, GST-cyclin A-Cdk2, and GST-Cdk4-cyclin D1, D2, or D3 were purified after overexpression in insect cells and tested for association with bacterially expressed p21 or deletion derivatives lacking either the Cy1 site (p21 Δ 17-24) or the K site (p21 Δ 53-58) (Fig. 2A). Deletion of the Cy1 site selectively disrupted association with cyclin A, cyclin E, and cyclin D-Cdk4 but did not disrupt association with cyclin A-Cdk2 and cyclin E-Cdk2. Deletion of the K site (Δ 53-58) did not affect association with the individual cyclins or cyclin-Cdk complexes.

To further substantiate this observation, GST-cyclin E-Cdk2 complexes were mixed with bacterially produced p21 and its derivatives, and p21-associated complexes were immunoprecipitated with the anti-p21 monoclonal antibody CP68 (which recognizes amino acids 130 to 150 of p21). Coimmunoprecipitated GST-cyclin E was detected by immunoblotting with a monoclonal antibody against cyclin E (HE12) (Fig. 2B). Consistent with the hypothesis that cyclin E-Cdk2 interacts with

p21 through either the Cy1 or the K site, this interaction was disrupted only when both sites were mutated (GST-p21 Δ 17-24, Δ 53-58).

Therefore, although discovered because of its ability to bind free cyclin E or A, the Cy1 site of p21 is used in interaction with cyclin-Cdk complexes. The Cy1 site is particularly important for association with cyclin D1-Cdk4. The Cy1 or K site independently permits the association of p21 with cyclin E-Cdk2.

A 12-amino-acid peptide containing the Cy1 site is sufficient to interact with cyclin E-Cdk2 or cyclin A-Cdk2. We tested whether a peptide, PS100, containing only the Cy1 site of p21 (residues 17 to 28, ACRRFLFGPVDSE) was sufficient to interact with cyclin-Cdk complexes (results summarized in Table 1). Since the cyclins produced by in vitro transcription and trans-

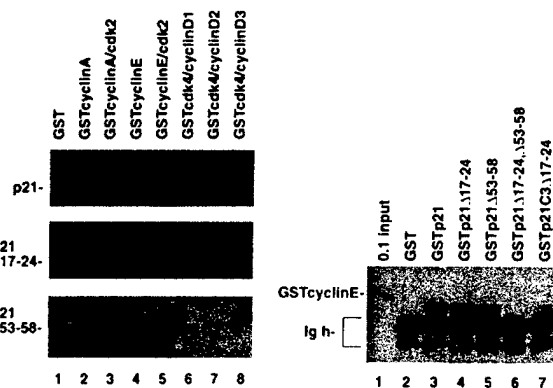


FIG. 2. (A) Association of cyclins or cyclin-Cdk complexes with p21 and its derivatives. All cyclins and cyclin-Cdk complexes were purified on glutathione-agarose beads, using insect cell lysates infected (or coinfecting) with corresponding baculoviruses. Proteins (p21 and its derivatives) bound to indicated GST fusion proteins after incubation with *E. coli* lysate containing recombinant human p21, p21 Δ 17-24, or p21 Δ 53-58 were visualized by immunoblotting with polyclonal anti-p21 antibody. (B) Double deletion of Cy1 and K sites (GST-p21 Δ 17-24, Δ 53-58) or of Cy1 and Cy2 sites (GST-p21C3 Δ 17-24) on p21 abolished its association with GST-cyclin E-Cdk2. Purified GST-cyclin E-Cdk2 protein was mixed with GST and GST-p21 derivatives indicated at the top. The p21-associated proteins were immunoprecipitated with antibody CP68 (anti-p21) and immunoblotted with anti-cyclin E antibody HE12. The bands below the GST-cyclin E band were immunoglobulin heavy chains (Ig h) in the immunoprecipitates.

TABLE 1. Association of p21 with several cyclin-Cdk complexes through the cyclin-binding motif at amino acids 17 to 24^a

Protein	Immunoprecipitation with antibody to:	Association with indicated form of p21					
		Wild type	Δ 17-24	Δ 53-58	Δ 53-58 + PS100	Δ 53-58 + PS101	Δ 17-24, Δ 53-58
Cdk2	Cdk2	+	+	—	ND	ND	—
Cdk4	ND	—	ND	ND	ND	ND	ND
Cyclin E	ND	+	—	+	ND	ND	ND
Cyclin E-Cdk2	Cdk2	+	+	+	—	+	—
Cyclin A-Cdk2	Cdk2	+	+	+	—	+	—
Cyclin D1-Cdk4	Cdk4	+	—	+	+	+	—

^a Two methods were used to generate the data. In the first method, radiolabeled Cdks or cyclins were synthesized by *in vitro* transcription-translation in rabbit reticulocyte lysates (Fig. 3). Cyclins E, A, and D1 were predominantly associated with the indicated rabbit Cdks, as evidenced by immunoprecipitation with the relevant anti-Cdk antibodies. A peptide containing the Cy2 site (PS102) gave results identical to those for PS100, while the mutant peptide (PS103) gave results identical to those for PS101. In the second method, cyclin-Cdk complexes were produced in insect cells by overexpression with a baculovirus expression system (see Table 2) and purified on glutathione-agarose columns (Fig. 2). ND, not done.

lation were associated with rabbit Cdks (as shown in Fig. 3), we first tested their binding to p21 Δ 53-58 (no K site). PS100 peptide competitively inhibited the association of cyclin E-Cdk2 (or cyclin A-Cdk2) with p21 Δ 53-58 (Fig. 4A), while a mutant peptide (FG mutated to KK; PS101) had no effect. Therefore, a peptide with the Cy1 site alone is sufficient to interact with cyclin E-Cdk2 and cyclin A-Cdk2 and competitively inhibit the association of cyclin-Cdks to a p21 molecule lacking a K site.

Surprisingly, the PS100 peptide also inhibited the association of cyclin E-Cdk2 and cyclin A-Cdk2 with wild-type p21, while the mutant PS101 peptide failed to do so (identical to the result shown in Fig. 4A). This result is contradicted by the robust association of p21 Δ 17-24 with cyclin E-Cdk2 (Fig. 2A, lane 5; Fig. 2B, lane 4) or cyclin A-Cdk2 (Fig. 2A, lane 3). The apparent contradiction can be explained if the p21 protein has a second redundant cyclin-binding site (Cy2 site). PS100 would inhibit interaction of the cyclin-Cdk complex with either the Cy1 or the Cy2 site, but deletion of only the Cy1 site in p21 would leave the Cy2-cyclin interaction unimpaired. This putative Cy2 site should have low affinity for isolated cyclin subunits; otherwise, deletion of Cy1 site will not disrupt the association of p21 with isolated cyclin E or cyclin A (Fig. 1C and 2A).

Cy2 site is present in the C terminus of p21. A second sequence (Cy2) with similarity to the Cy1 site was, in fact, iden-

tified at residues 152 to 158 of p21 (HSKRRLIE; the underlined sequence is most similar). To test whether Cy2 is really a cyclin-binding site, PS102, a peptide containing residues 150 to 161 of p21, was used to compete for the association of cyclin E-Cdk2 with p21 Δ 53-58. PS102 competitively inhibited the association of cyclin E-Cdk2 with p21 Δ 53-58 in a manner analogous to that for PS100, while a mutant peptide, PS103 (RL residues changed to DD), did not (Fig. 4A; Table 1). Like

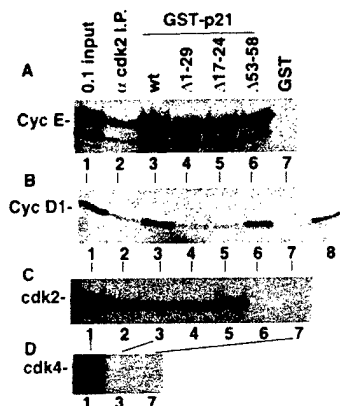


FIG. 3. Association of p21 and its derivatives with cyclin (Cyc) E, cyclin D1, Cdk2, and Cdk4. All of the cyclins and Cdks were produced by *in vitro* transcription and translation using rabbit reticulocyte lysate. The association of cyclins with rabbit Cdks was verified by immunoprecipitation (I.P.) using a polyclonal antibody against Cdk2 (lane 2) or Cdk4 (lane 8 in panel B). One-tenth-input, radioactive translation products bound to indicated GST fusion proteins or antibodies were visualized by fluorography. wt, wild type.

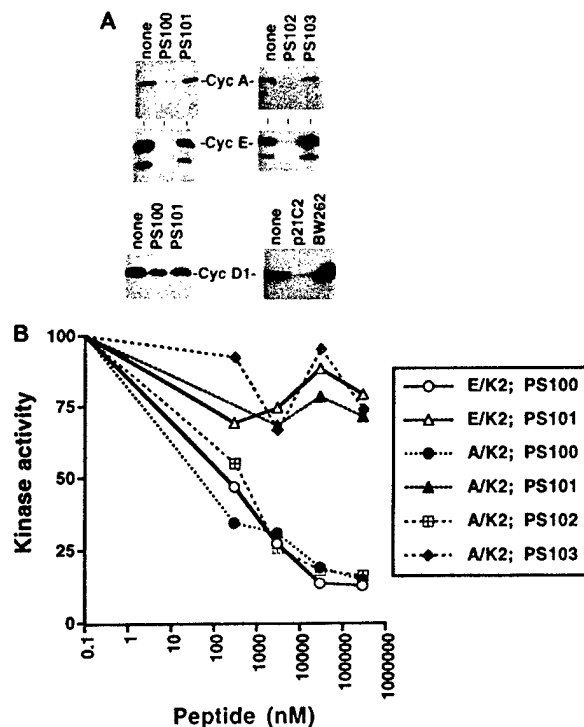


FIG. 4. (A) Association of GST-p21 Δ 53-58 with cyclin (Cyc) A-Cdk2 or cyclin E-Cdk2 is competitively inhibited by PS100 peptide (Cy1 site) or PS102 peptide (Cy2 site) but not by mutant peptide PS101 or PS103. The same results are obtained with GST-p21. Association of cyclin D1-Cdk4 with GST-p21 Δ 53-58 was not blocked by PS100 or PS102 but was blocked by a longer peptide containing the Cy2 site (p21C2). The cyclin-Cdk complexes were made by *in vitro* transcription-translation as described in the footnote to Table 1, and association with GST-p21 Δ 53-58 was tested in the presence of 75 μ M indicated peptides. BWH262 is a negative control peptide described in Materials and Methods. Bound radioactive cyclins were visualized by fluorography. (B) Wild-type peptide PS100 inhibits the kinase activity of cyclin E-Cdk2 (E/K2), and both PS100 and PS102 inhibit the kinase activity of cyclin A-Cdk2 (A/K2). The mutant peptides PS101 and PS103 do not inhibit either kinase (see Table 2 for details).

TABLE 2. Comparison of derivatives of p21 with all or a subset of cyclin- or Cdk-binding sites for the ability to inhibit cyclin-Cdk kinases^a

Protein	Binding site on p21			K_{iapp}^b		
	Cy1	K	Cy2	Cyclin E-Cdk2	Cyclin A-Cdk2	Cyclin D1-Cdk4
p21	+	+	+	1	0.1	8.5
p21Δ17-24	—	+	+	4.3	0.4	>850 ^c
p21Δ53-58	+	—	+	87	35	30
p21N	+	+	—	4	0.2	11
p21NΔ17-24	—	+	—	88	3.4	>1,150 ^c
p21C3Δ17-24	—	+	—	190	2.8	>952 ^c
Peptide PS100	+	—	—	296	220	>300,000 ^c
Peptide PS102	—	—	+	32,000	800	>300,000 ^c
Peptide p21C2	—	—	+	6,500	90	2,000

^a Cyclin-Cdk complexes were produced in insect cells by overexpression with recombinant baculoviruses and purified on glutathione-agarose columns; kinase activity was tested in vitro, using bacterially produced GST-RbC as the substrate. The phosphorylation of substrate was quantitated both by liquid scintillation counting of bands excised from gels and by PhosphorImager analysis. Cy1, Cy2, and K are described in the text and Fig. 10. The synthetic peptides are described in the text. The p21 proteins were all produced and purified as GST fusion proteins in bacteria. p21N, amino acids 1 to 90 of p21; p21C3, deletion of amino acids 150 to 164 of p21 (Cy2 site); Δ17-24, deletion of amino acids 17 to 24 (Cy1 site); Δ53 to 58, deletion of amino acids 53 to 58 (K site).

^b Concentration of inhibitor which inhibits kinase activity to 50% of activity seen in the absence of any inhibitor.

^c Inhibition to 50% of basal activity is not achieved even at the highest concentration of inhibitor tested (e.g., 850 nM).

PS100, PS102 specifically disrupted the interaction of wild-type p21 with cyclin E-Cdk2 (data not shown).

If Cy2 is responsible for the association of p21Δ17-24 with cyclin E-Cdk2 (Fig. 2A, lane 5; Fig. 2B, lane 4), we predict that a p21 molecule missing both Cy1 and Cy2 sites but retaining an intact K site would fail to associate with cyclin E-Cdk2. Consistent with this prediction, GST-p21Δ17-24 (containing amino acids 1 to 150 of p21 with the deletion of the Cy1 site) or GST-p21NΔ17-24 failed to associate with cyclin E-Cdk2 (Fig. 2B, lane 7, and data not shown). Therefore, at least one Cy site is required for the stable interaction of p21 with cyclin-Cdk complexes.

Although the failure of GST-p21Δ17-24 to associate with cyclin D1-Cdk4 (Fig. 3B, lane 5) indicates the primary importance of the Cy1 site in the association of p21 with this complex, PS100 (Cy1 peptide) and PS102 (Cy2 peptide) were unable to block the cyclin D1-Cdk4-p21 interaction (Fig. 4A and data not shown). One explanation is that the synthetic peptides were too small to contain the entire cyclin D1-interacting motif. This explanation is supported by the observation that p21C2, a longer peptide containing residues 127 to 164 of p21 (which includes the Cy2 site), efficiently blocked the association of cyclin D1-Cdk4 with p21 (Fig. 4A). Therefore, the exact nature of the interaction of the cyclin-binding motifs of p21 may be subtly different with different cyclin-Cdk complexes.

The Cy regions of p21 alone weakly inhibit the kinase activity. We tested whether peptides containing the Cy regions of p21 inhibited kinase activity. As the substrate we used GST-RbC, a recombinant protein containing the C-terminal portion of the retinoblastoma gene product, a physiological substrate for the G₁ cyclin-Cdk complexes. Peptides PS100 (Cy1 site) and PS102 (Cy2 site) inhibited cyclin E-Cdk2 or cyclin A-Cdk2 kinase activities (Fig. 4B; Table 2), while mutant PS101 and PS103 peptides did not. The Cy1 peptide was a better inhibitor than the Cy2 peptide, consistent with the greater avidity of the

Cy1 site for the cyclins. The Cy peptides were far weaker than intact p21 or p21 with one K site and at least one Cy site intact (see below and Table 2). However, inhibition by the wild-type Cy peptides was significant since the mutant Cy peptides PS101 and PS103 did not inhibit the kinase to 50% of control even at concentrations exceeding 300 μM.

PS100 and PS102 did not inhibit cyclin D1-Cdk4, as expected from the observation that they were insufficient to associate with the kinase complex. p21C2, the longer Cy2-containing peptide capable of associating with cyclin D1-Cdk4, inhibited the kinase activity (Table 2).

The Cy regions of p21 are essential for kinase inhibition. The K site of p21 is essential for kinase inhibition (Table 2; also see reference 30). Because the Cy1 and Cy2 regions are important for the association of p21 with cyclin-Cdk complexes (Table 1), we expect them to be similarly important for kinase inhibition. To test this, the K_{iapp} s of mutant p21 derivatives were determined (Table 2).

Loss of the Cy1 site of p21 or p21N abolished the ability to inhibit cyclin D1-Cdk4 kinase. These results are consistent with the absolute requirement for the Cy1 site for mediating p21-cyclin D1-Cdk4 association. For cyclin A-Cdk2 or cyclin E-Cdk2, deletion of the Cy1 site alone (p21Δ17-24) increased K_{iapp} fourfold compared with wild-type p21. A more dramatic effect was observed when both Cy1 and Cy2 sites were deleted simultaneously (compare p21Δ17-24 with p21NΔ17-24 or with p21C3Δ17-24). These results are consistent with the observation that either the Cy1 or the Cy2 site stabilizes the interaction with cyclin E or A in the cyclin-Cdk2 complexes, and deletion of both Cy sites destabilizes the interaction (Fig. 2B, lane 7).

Because of the surprising result that a p21 derivative containing an intact K site (p21NΔ17-24) failed to inhibit the kinase, we made a quantitative assessment of its binding to the isolated Cdk2 subunit (Fig. 5). As mentioned earlier, Cdk2 produced by in vitro transcription and translation in a rabbit reticulocyte lysate is free of cyclins. p21NΔ17-24 bound quantitatively to Cdk2 (Fig. 5A). However, association with cyclin E produced in a similar lysate (free cyclin E and cyclin E complexed with rabbit Cdk2) was impaired by the Cy1 deletion (Fig. 5B). Therefore, the loss of kinase inhibition by p21NΔ17-24 is due to the loss of interaction of the inhibitor with the cyclin in the cyclin-Cdk complex.

The Cy sites of p21 are important for suppression of *Xenopus* DNA replication. If the Cy deletion derivatives of p21 lose the

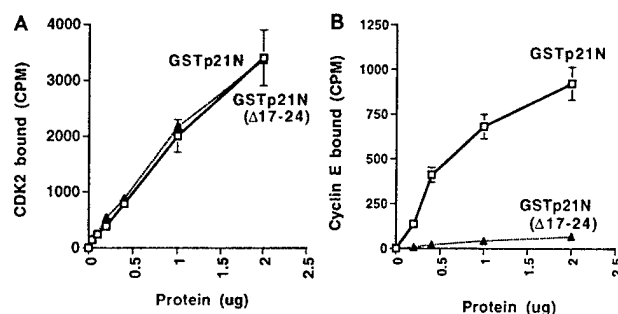


FIG. 5. Deletion of the Cy1 site in p21N abolishes its interaction with cyclin E or cyclin E-Cdk2 complex without affecting its interaction with isolated Cdk2 subunit. Cyclin E and Cdk2 were synthesized with [³⁵S]methionine by in vitro transcription and translation, using rabbit reticulocyte lysate. Various amounts of GST-p21N or GST-p21NΔ17-24 were incubated with lysates containing either cyclin E or Cdk2. Proteins associated with GST-p21N or GST-p21NΔ17-24 were pulled down by using glutathione-agarose beads, visualized by fluorography, and quantitated by counting excised gel slices. Averages of two to three experiments are presented, with standard deviations as the error bars.

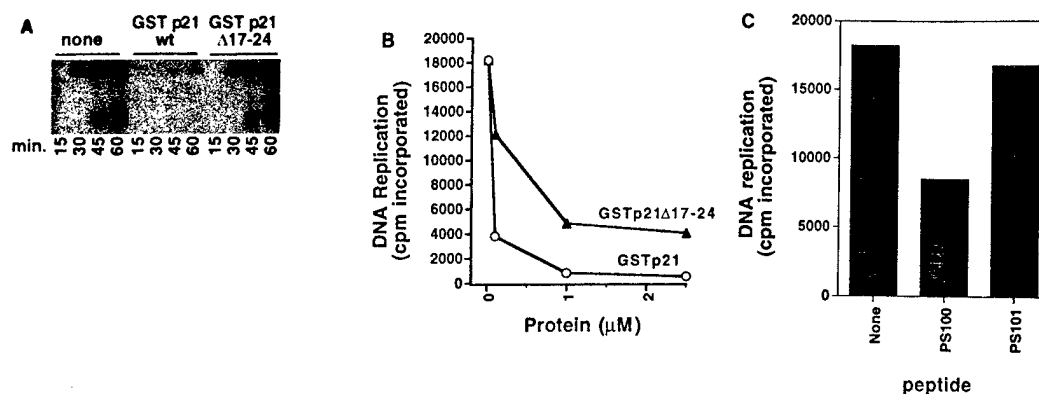


FIG. 6. The Cy1 site is important for optimal inhibition of DNA replication in *Xenopus* egg extracts. Replication of sperm DNA in *Xenopus* egg extracts (with radiolabeled α - 32 PdATP) were carried out in the absence or presence of the indicated proteins or peptides. In panel A, the reactions (with 1 μ M proteins indicated) were stopped at various time points, and products were visualized by autoradiography. wt, wild type. In panels B and C, the reactions were stopped at 2 h, and the products were quantitated by counting excised gel slices.

ability to interact with and inhibit cyclin-Cdk kinases, these mutations should affect the biological activities of p21. To test this, we assayed DNA replication in *Xenopus* egg extracts, which are sensitive to the Cdk inhibitory activity of p21 (17). GST-p21 or GST-p21Δ17-24 was titrated into the *Xenopus* egg extracts (Fig. 6A and B). Loss of the Cy1 site decreased replication inhibition fivefold (50% inhibitory concentration of GST-p21 = 0.1 μ M; 50% inhibitory concentration of GST-p21Δ17-24 = 0.5 μ M). Both proteins associated with *Xenopus* Cdk2 and were equally stable under replication conditions (data not shown).

We also tested whether the peptide containing a Cy1 motif (PS100) was sufficient to inhibit DNA replication (Fig. 6C). PS100 peptide at a concentration of 3 mM inhibited DNA replication to 47% of the control reaction level, while the mutant peptide PS101 permitted replication to 92% of the control reaction level. The high concentration of PS100 required to inhibit the replication reaction is consistent with the weak inhibition of cyclin-Cdk kinases by the peptide (Table 2).

The Cy sites of p21 are required for cell growth suppression. To determine whether the Cy sites were important for the biological activity of p21 in vivo, we tested cell growth suppression by various deletion derivatives of p21 (3). Plasmids expressing p21⁺ (Cy1⁺ K⁺ Cy2⁺), p21Δ17-24 (Cy1⁻ K⁺ Cy2⁺), p21N (Cy1⁺ K⁺ Cy2⁻), and p21NΔ17-24 (Cy1⁻ K⁺ Cy2⁻) were stably transfected into SaOs2 cells (p53 null, Rb null) and H1299 cells (p53 null, intact Rb), and colony formation was measured (Fig. 7A). In agreement with the in vitro results, only p21 with neither the Cy1 nor the Cy2 site (p21NΔ17-24) significantly lost growth suppression activity. Immunoblotting of transiently transfected cell extracts revealed that the steady-state level of p21NΔ17-24 was as high as that of the growth-suppressive p21 derivatives (Fig. 7B). Immunoprecipitation of the p21 derivatives followed by immunoblotting with anti-Cdk2 antibody showed that only p21NΔ17-24 failed to associate with Cdk2 (data not shown). Therefore, although p21NΔ17-24 associates with the isolated Cdk2 subunit (Fig. 1D, lane 10; Fig. 5B), the interaction of the K site alone with cyclin-Cdk2 kinase complex was too weak to be detected either in vitro (Fig. 2B) or in vivo (data not shown). However, addition of either Cy site stabilized the p21-cyclin-Cdk interaction and inhibited cell growth.

A monoclonal antibody that recognizes the Cy1 site disrupts the association of p21 with cyclin E-Cdk2 and cyclin D-Cdk4 but not cyclin A-Cdk2. We expect the Cy sites to be important

for the formation of p21-cyclin-Cdk complexes in vivo at a physiological concentration of p21. CP36 is a monoclonal antibody that fails to recognize p21Δ17-24, indicating that its epitope overlaps the Cy1 region of p21 (Fig. 8A). Preincubation of p21 with this antibody selectively disrupts the association of recombinant GST-cyclin E-Cdk2 with p21 in a pull-down assay (Fig. 8A). However, the association of p21 with GST-cyclin A-Cdk2 is not affected by the antibody. CP68, an antibody recognizing the C terminus of p21, and CP2, an antibody recognizing the N-terminal 17 amino acids of p21, did not block the association of p21 with cyclin E-Cdk2 or cyclin A-Cdk2 (Fig. 8A and data not shown). As cyclin A-Cdk2 and cyclin E-Cdk2 have the same Cdk2 subunit, the differential effect of CP36 on the association of p21 with either cyclin A-Cdk2 or cyclin E-Cdk2 confirms that the antibody disrupts an interaction between the cyclins and p21.

CP36 antibody allowed us to test whether the Cy1 site is important for p21-cyclin-Cdk complex formation in cells. CP36, CP68, and CP2 antibodies were used to immunoprecipitate p21 from cell extracts, and coprecipitated cyclins and Cdks were detected by immunoblotting (Fig. 8B). CP36 specifically failed

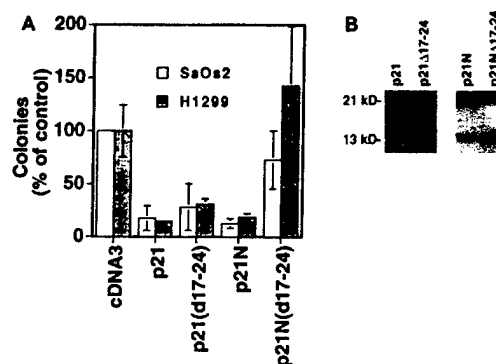


FIG. 7. (A) Inhibition of cell growth in SaOs2 cells (p53⁻ Rb⁻) and H1299 cells (p53⁻ Rb⁺). Shown are the numbers of stably transfected colonies obtained by transfection of vector alone (cDNA3) and vectors expressing indicated alleles of p21 and p21N (d = Δ). The results are expressed as percentages of the vector control level, with the means and standard deviations of 9 to 12 (SaOs2) and 3 to 4 (H1299) independent transfections. (B) Expression levels of the Δ17-24 and wild-type alleles were comparable after transient transfection of the corresponding plasmids into H1299 cells, as measured by immunoblotting of transfected cell lysates (48 h after transfection) with anti-p21 antibody.

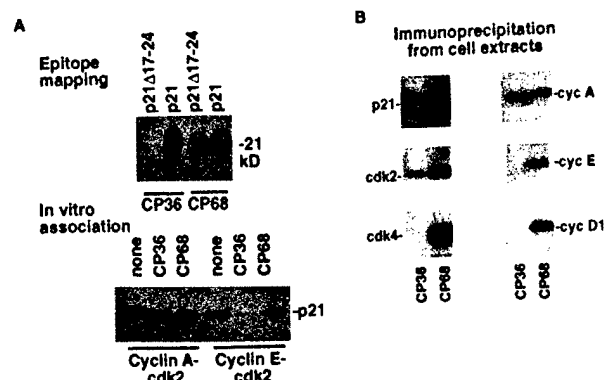


FIG. 8. (A) For epitope mapping, 100 ng of bacterially expressed p21 or p21Δ17-24 was immunoblotted with antibodies CP36 and CP68. CP68 recognizes the 130–150 region and CP2 recognizes the 1–17 region of p21 (data not shown). To analyze in vitro association, bacterially produced p21 (200 ng) was preincubated on ice with 50 μl of hybridoma culture supernatants containing no antibody, CP36, or CP68 for 30 min and then incubated with indicated GST-cyclin-Cdk2 complexes (1 μg) bound to glutathione-agarose beads (standard pull-down conditions as for Fig. 1). p21 bound to the beads was visualized by immunoblotting with anti-p21 monoclonal antibody CP36. (B) Immunoprecipitation from cell extracts. Equal amounts of WI38 cell extracts were immunoprecipitated with antibody CP36 or CP68 (covalently coupled to beads), and the precipitates were immunoblotted to detect the indicated proteins. cyc, cyclin.

to coimmunoprecipitate cyclin E, cyclin D1, and Cdk4 protein with p21 from cell extracts, confirming that the Cy1 region of p21 is used in vivo for interaction with these proteins. Cyclin A and Cdk2 were present in the CP36 immunoprecipitates, in agreement with in vitro association results shown in Fig. 8A.

Association of p21 with cyclin-Cdk through a Cy1 site allows partially active cyclin-Cdk kinase to be associated with p21. Surprisingly, active cyclin-Cdk is found complexed with the Cdk inhibitor p21 in cell extracts (15, 46). Our results suggest that complete kinase inhibition requires association of the cyclin-Cdk complex with at least one Cy site and the K site of p21. However, stable association can occur with p21 containing only Cy sites and no K site. Therefore, it is conceivable that partially active cyclin-Cdk kinase may be complexed with p21 through the Cy sites.

To test this hypothesis, we mixed wild-type p21, p21Δ17-24 (ΔCy1), and p21Δ53-58 (ΔK) with GST-cyclin E-Cdk2 and immunoprecipitated the p21-cyclin-Cdk complexes with the anti-p21 antibody CP68. Equal amounts of GST-cyclin E were detected in the three immunoprecipitates by immunoblotting (Fig. 9, left bottom). However, the only immunoprecipitate containing active kinase was the one with p21Δ53-58 (ΔK) (Fig. 9, left top). The association was selectively disrupted by the PS100 peptide (Fig. 9, right). Therefore, partially active cyclin E-Cdk2 kinase stably associates with p21 through the Cy sites.

Addition of wild-type p21 (Cy1⁺ K⁺ Cy2⁺) to the immunoprecipitate of p21Δ53-58-cyclin E-Cdk2 inhibited, but p21Δ17-24 (Cy1⁻ K⁺ Cy2⁺) failed to inhibit, the residual kinase activity (data not shown). Therefore, the K site cannot be provided in *trans* to cyclin-Cdk2 already complexed with p21 through the Cy sites. We have not observed any additive inhibition when GST-p21Δ17-24 (K site alone) and PS100 peptide (Cy1 site alone) were added simultaneously to a kinase reaction (data not shown). Thus, there is no evidence yet that the Cy and K activities can be provided in *trans* by two different p21 molecules.

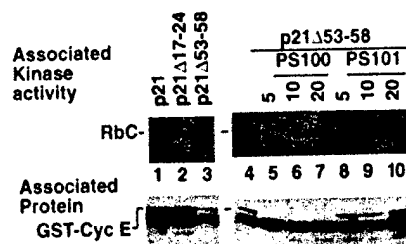


FIG. 9. Association with p21 through only the cyclin subunit allows partially active kinase to be complexed with p21. To assay associated kinase activity, wild type, Δ17-24, or Δ53-58 p21 (mutated in the K site) was mixed with GST-cyclin E-Cdk2 and immunoprecipitated with antibody CP68, and the activity of the kinase in the precipitate was tested on GST-RbC. For the panel on the right, p21Δ53-58 was mixed with cyclin E-Cdk2 kinase in the presence of no peptide (lane 4), the wild-type PS100 peptide (lanes 5 to 7), or the mutated PS101 peptide (lanes 8 to 10) added at the amounts indicated on top (micrograms per 200 μl). To analyze associated proteins, the presence of equivalent amounts of GST-cyclin E (lanes 1 to 4 and 8 to 10) and absence of GST-cyclin E (lanes 5 to 7) in the immunoprecipitates was verified by immunoblotting with anti-cyclin E antibody HE12. Immunoblotting with anti-Cdk2 antibody showed that Cdk2 levels paralleled that of cyclin E (data not shown). The bands below GST-cyclin (Cyc) E are immunoglobulin heavy chain present in the immunoprecipitates.

DISCUSSION

We have shown that the interaction of p21 with cyclin A-Cdk2 or cyclin E-Cdk2 kinases involves three sites: Cy1, Cy2, and K. Cy1 and Cy2 are redundant sites that bind cyclins, and the K site binds Cdk2 (Fig. 10). Interaction of p21 with cyclin D1-Cdk4 depends exclusively on the Cy1 site.

The Cy1 site in p21 was identified by using cyclin A or E

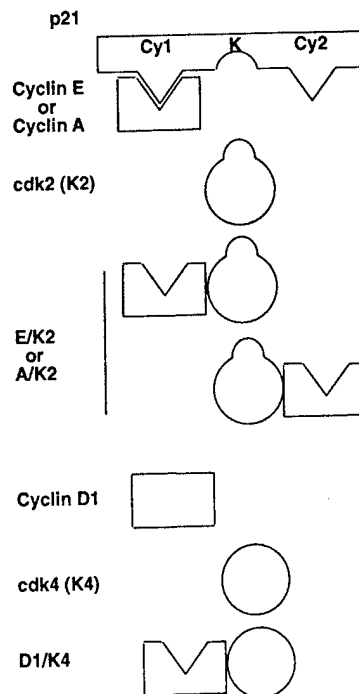


FIG. 10. Summary of the interactions presented in the report. p21 has two cyclin-binding sites, Cy1 and Cy2, and one site for Cdk2 (K site). Isolated cyclin A and cyclin E associate with the Cy1 site, and Cdk2 associates with the K site. With cyclin A-Cdk2 or cyclin E-Cdk2, p21 utilizes the Cy1 or Cy2 site for association with cyclin and the K site for association with Cdk2. Isolated cyclin D or Cdk4 does not associate with p21. The cyclin D-Cdk4 complex exclusively utilizes the Cy1 site for interaction with p21.

expressed in bacteria or insect cells (Fig. 1A, 1C, and 2A). In vitro, the Cy1 site did not interact with free Cdk2. We have also shown with peptide competition and antibody blocking that the Cy1 site is utilized for the association of p21 with cyclin-Cdk complexes. We therefore propose that even in the cyclin-Cdk complex, the Cy1 site of p21 interacts with the cyclin, and the K site interacts with the Cdk. The crystal structure of cyclin A-Cdk2 (18) suggests that a protein like p21 could easily bind to both the cyclin and Cdk molecules simultaneously. The existence of both the cyclin-binding Cy1 motif and the Cdk-binding K motif on p21 may also explain why p21 facilitates the association of cyclins and Cdks (15).

There are two copies of the cyclin-binding motif in p21. The importance of the Cy2 site is still unclear. Cy2 is a low-affinity site for cyclins in comparison with Cy1. While the Cy1-cyclin interaction is avid enough to be detected by pull-down assay, the Cy2-cyclin interaction can only be inferred from competition for binding and kinase inhibition assays. Since the Cy2 site overlaps the PCNA binding site of p21 (9), we examined the effect of PCNA on the ability of p21 Δ 17-24 (deleted Cy1 site) to bind cyclin E-Cdk2 and inhibit its activity in vitro. While PCNA did not affect the ability of wild-type p21 to bind and inhibit cyclin E-Cdk2, it diminished that of p21 Δ 17-24 (data not shown). This finding raised the possibility that the Cy2 site may not be used in cells for interaction with cyclin-Cdk complexes since this site may be occupied by PCNA in vivo. When p21 is induced in response to DNA damage, during differentiation, or experimentally by transfection (Fig. 7A), the Cy2 site of p21 may exceed that of PCNA in specific subcellular compartments and thus become available to interact with cyclin-Cdk complexes. Furthermore, by competing with cyclins for the Cy2 site in p21, PCNA may regulate the activity of p21 on cyclin-Cdks.

While the deletion of the Cy1 site of p21 in this study did not disrupt its interaction with cyclin E-Cdk2 complexes, Luo et al. (27) and Lin et al. (26) have reported that mutations of the Cy1 site in p27 and p21 dramatically decrease or abolish interaction between p21 or p27 with cyclin E-Cdk2. The apparent discrepancy could be because both of these groups used cyclin E-Cdk2 in crude extracts. First, a significant fraction of the cyclin E in these extracts (particularly that obtained by in vitro transcription-translation) may not be complexed with Cdk2. As shown in our study, deletion of the Cy1 site disrupts the interaction between p21 and free cyclin E but not that of p21 and cyclin E-Cdk2. Second, as PCNA competes with cyclin-Cdk complexes for the C-terminal region (Cy2 site) of p21, the relative concentration of PCNA in crude extracts could determine whether p21 without a Cy1 site binds stably to cyclin E-Cdk2.

Deletion of the K site (p21 Δ 53-58) increases K_{iapp} for cyclin E-Cdk2 90-fold, as does the deletion of both Cy sites (p21 Δ 17-24 or p21C3 Δ 17-24) (Table 2). Deletion of the K site increases the K_{iapp} for cyclin A-Cdk2 340-fold, while deletion of both Cy sites increases the K_{iapp} 34-fold (Table 2). Thus, the Cy sites are as important as the K site for inhibiting cyclin E-Cdk2 but not cyclin A-Cdk2. Likewise, CP36 antibody disrupts the p21-cyclin E-Cdk2 complex but not the p21-cyclin A-Cdk2 complex. Therefore, the Cy site of p21 appears more important for association with cyclin E-Cdk2 than for association with cyclin A-Cdk2.

The interaction of p21 with the cyclin D-Cdk4 complex is much stronger than that obtained with the individual subunits. Also in contrast to the kinases described above, the interaction is totally dependent on an intact Cy1 site. On the basis of our results with cyclins E and A, we postulate that it is the cyclin D1 subunit in the cyclin-Cdk complex which executes the critical interaction with the Cy motif of p21.

Why is the Cy site important for kinase inhibition? The Cy-cyclin association stabilizes the interaction of the K site with Cdk2, as suggested by the failure of cyclin-Cdk complexes to associate stably with p21 containing only the K site (Fig. 2B). The cyclin-Cy interaction may be the first step in p21-cyclin-Cdk complex formation, which then facilitates the second step, whereby the K site associates with and inhibits the Cdk. Thus, one role of the Cy site could be to stabilize or promote the association of the K site with Cdk2. However, kinase inhibition by an isolated Cy site implies that the Cy-cyclin association directly inhibits a step in the catalytic process. Perhaps the physiological substrates of cyclin-Cdk kinases also use a two-step mechanism to interact with cyclin-Cdks whereby they first loosely associate with cyclins before engaging with the catalytic site of the cdk. The Cy peptides could partially inhibit kinase activity by blocking the first step of the reaction. Indeed, as discussed below, several putative substrates of G₁ cyclin-Cdk kinases associate stably with cyclin-Cdk kinases, utilizing motifs that resemble the Cy sequences. This model is supported by the observation that the Cy peptides are poor kinase inhibitors with some substrates, e.g., histone H1 (unpublished data; also see reference 2).

If simultaneous interaction of the Cy1 site with cyclin and the K site with Cdk is essential for optimal kinase inhibition, situations in which only one site is utilized will result in partially inhibited kinase being complexed with p21. We demonstrated that such a situation could be simulated in vitro with a version of p21 with a deletion of the K site. Thus, the presence of partially inhibitory, weakly interacting modules on p21 probably explains how active kinase is found complexed with p21 in immunoprecipitates of cell extracts.

Addition of extra p21 to the active kinase-p21 complex in Fig. 9 quenched residual kinase activity completely. Similar observations have been interpreted to indicate that the stoichiometry of the p21-cyclin-Cdk interaction is important for optimal kinase inhibition (15, 46), with two molecules of p21 required per cyclin-Cdk complex for total kinase inhibition. We wondered whether the requirement for two p21 molecules for optimal kinase inhibition is explained by one molecule interacting with the Cdk through the K site and the other interacting with cyclin through a Cy site, but we did not obtain any evidence that the two sites can be provided in *trans* by two different molecules. Alternatively, the kinase quenching seen with addition of more wild-type p21 (both in Fig. 9 and in the literature) could be due to the dissociation of cyclin-Cdk from partially inhibitory complexes utilizing only Cy-cyclin contacts, followed by association with a new p21 molecule through both Cy-cyclin and K-Cdk contacts.

The core of the Cy motif in p21 appears to be two basic residues followed by two nonpolar residues (RRLF or RRLI), although further mutagenesis is required to determine the exact nature of this motif and the contribution of flanking sequences. Sequences similar to the Cy motif described here are critical for the association of cyclin E-Cdk2 with p27 (27) and of cyclin A-Cdk2 with p107 (47) or with E2F1 (22). None of these studies demonstrated that the interaction occurred with the cyclin subunit of the cyclin-Cdk complex. We suggest that this conserved Cy motif of p27, p107, and E2F1 interacts specifically with cyclin E or promotes complex formation. Also, as discussed above, in some cases (p107 and E2F1), the Cy motif may be an accessory site to target the substrate to a cyclin-Cdk kinase.

While preparing this report, we learned that Lin et al. had analyzed the activity of p21 by mutagenesis (26), while Chen et al. had analyzed the activity of p21 with synthetic peptides (2). Their results agree with ours in defining the importance of the

Cy1 site. Lin et al. (26) defined the cyclin-binding motif by using *in vitro*-translated cyclins, which we have shown are complexed with rabbit Cdks. Chen et al. (2) demonstrated that a peptide containing what we term the Cy1 or K site interferes with the association of cyclin-Cdk with p21. In this report, we have clearly demonstrated that the Cy site interacts with cyclin in the total absence of Cdks by using bacterially expressed cyclins. We have also determined a second Cy site on p21. In addition, we show that the Cy sites (i) stabilize the interaction of the K site with cyclin-Cdk and (ii) inhibit kinase activity directly. Finally, experiments with the CP36 antibody and *Xenopus* DNA replication reactions add to the experimental evidence that the Cy1 motif of p21 is important at physiological conditions. Taken together, these three reports clearly demonstrate the existence of cyclin-binding motifs in p21 and their necessity for its biological function.

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p21^{Cip1/Waf1} disrupts the recruitment of human Fen1 by proliferating-cell nuclear antigen into the DNA replication complex

(cell cycle/maturation factor I/DNA repair)

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ABSTRACT Fen1 or maturation factor 1 is a 5'–3' exonuclease essential for the degradation of the RNA primer–DNA junctions at the 5' ends of immature Okazaki fragments prior to their ligation into a continuous DNA strand. The gene is also necessary for repair of damaged DNA in yeast. We report that human proliferating-cell nuclear antigen (PCNA) associates with human Fen1 with a K_d of 60 nM and an apparent stoichiometry of three Fen1 molecules per PCNA trimer. The Fen1–PCNA association is seen in cell extracts without overexpression of either partner and is mediated by a basic region at the C terminus of Fen1. Therefore, the polymerase δ –PCNA–Fen1 complex has all the activities associated with prokaryotic DNA polymerases involved in replication: 5'–3' polymerase, 3'–5' exonuclease, and 5'–3' exonuclease. Although p21, a regulatory protein induced by p53 in response to DNA damage, interacts with PCNA with a comparable K_d (10 nM) and a stoichiometry of three molecules of p21 per PCNA trimer, a p21–PCNA–Fen1 complex is not formed. This mutually exclusive interaction suggests that the conformation of a PCNA trimer switches such that it can either bind p21 or Fen1. Furthermore, overexpression of p21 can disrupt Fen1–PCNA interaction *in vivo*. Therefore, besides interfering with the processivity of polymerase δ –PCNA, p21 also uncouples Fen1 from the PCNA scaffold.

The eukaryotic DNA replication machinery has been extensively defined by biochemical fractionation of cell extracts and by genetic analysis in yeast. DNA polymerase α –primase initiates RNA primers on both strands of DNA at the origin of replication, which are subsequently elongated as DNA strands (1–4). On both the leading and lagging strands, the initiator DNA laid down by DNA polymerase α is subsequently elongated by DNA polymerase δ . The switch between the two polymerases involves protein–protein interactions of the single-stranded DNA binding factor RPA, DNA polymerase α , RF-C, proliferating-cell nuclear antigen (PCNA), and DNA polymerase δ itself (ref. 5 and references therein). PCNA promotes the processivity of DNA polymerase δ allowing it to synthesize long strands of DNA necessary for replicating the leading strand. The presence of the proofreading exonuclease in DNA polymerase δ also suggests that this enzyme is best suited for replication with minimal errors. Due to the close similarity between the replication processes in prokaryotes and eukaryotes, it is somewhat surprising that the eukaryotic polymerases are missing in one crucial activity, the 5'–3' exonuclease (6). This becomes particularly important on the lagging strand, where as each new Okazaki fragment reaches the 5' end of the previous Okazaki fragment, a 5'–3' exonuclease is required. While RNase H can remove most of the RNA primer, the ribonucleotide–deoxyribonucleotide bond

requires the activity of a special 5'–3' exonuclease that was called maturation factor 1 (MF1) or Fen1 (5, 7–10).

MF1/Fen1 has been discovered in several different contexts. It was identified as a gene, *rad2*, that confers radiation sensitivity when mutated in *Schizosaccharomyces pombe* (11). Rad2 was found to be homologous to an anonymous open reading frame in *Saccharomyces cerevisiae*, YKL510 (renamed RAD27), that when mutated confers radiation sensitivity (12) and an instability in simple direct repeated sequences (13). The instability was primarily in the form of simple insertions, suggesting that an important repair mechanism in the replication process was missing. In yeast missing two G₁ cyclins, CLN1 and CLN2, mutations in the RAD27 resulted in a cell division cycle arrest consistent with a checkpoint being activated by incomplete DNA replication during S phase (12, 14). Yeast with a deletion of the RAD27 gene demonstrate increased chromosome loss and a temperature-sensitive cell division cycle block consistent with a requirement for the gene product in S phase. The Fen1 exonuclease has also been independently purified by virtue of its ability to endonucleolytically cleave a flap DNA, a putative intermediate in the process of recombination (15). A similar cleavage may also be important for removing insertion mutations created by the replication machinery.

Human PCNA, the processivity factor for DNA polymerase δ (16, 17), has a ring-shaped structure made up of three subunits that assembles around DNA like a “ring around a curtain-rod.” The structure suggests that its mechanism of action is to move along the DNA like a sliding clamp to which the polymerase δ is tethered (18, 19). Besides interacting with polymerase δ and RF-C, PCNA associates with the cell-cycle regulatory factor p21. p21 associates with PCNA through its C terminus, and with cyclin–cdks through its N terminus, thereby forming a bridge between the two protein complexes (20–24). By interacting with PCNA, p21 inhibits the activity of PCNA and disrupts the replication machinery. Surprisingly, this interaction does not inhibit excision repair, which also utilizes PCNA (25, 26). Since p21 is transcriptionally induced by p53 and since this pathway is activated after damage to DNA (27), the differential effect of p21 on the replication and repair functions of PCNA could be important for the pause in DNA replication observed after DNA damage allowing the damage to be repaired before it is “fixed” by replication.

In view of the important role of PCNA in DNA replication and repair, we searched for human gene products that interact with PCNA in a two-hybrid interaction. The results reported in this paper suggest that human MF1/Fen1 5'–3' exonuclease associates with human PCNA *in vivo* and *in vitro*. This association completes the DNA polymerase holoenzyme, because

the DNA polymerase δ -PCNA-Fen1 complex has all three activities found in prokaryotic DNA polymerases. Through this association, PCNA recruits Fen1 into replication complex and ensures the completion of DNA replication. These results may explain why mutations in the Fen1 subunit of the DNA polymerase holoenzyme result in incomplete DNA replication and in the creation of insertion-deletion mutants in simple direct repeats. p21 disrupts Fen1-PCNA interaction providing an additional mechanism by which p21 could inhibit DNA replication.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen. For the yeast two-hybrid screen, the human PCNA open reading frame was cloned by PCR and inserted into the pAS2 vector (28) to create a fusion protein with the GAL4 DNA-binding domain. pAS2-hPCNA was transformed into yeast strain Y190. For screening, a B-cell cDNA library in the pACT vector was transformed into Y190 strain containing pAS2-hPCNA.

Plasmid Construction and Protein Purification. pGST-PCNA was constructed by ligating the full PCNA coding sequence into pGEX-5x-3 (Pharmacia) between *Bam*HI and *Sal*I sites. pGST-p21C2 expresses the C-terminal 39 amino acids of p21 fused to glutathione *S*-transferase (GST) (29). pGST-Fen1C was constructed by ligating the sequence encoding the C-terminal amino acids of human Fen1 (residues 307–380, a *Bcl*II-*Sal*I fragment) into the pGEX-5x-3 (*Bam*HI and *Sal*I). Plasmids were transformed into *Escherichia coli* BL21; cell lysis and purification of GST fusion proteins were done as described (22).

The cDNA encoding the full-length human Fen1 was a gift from J. Murray (University of Sussex, U.K.). pET-Fen1 was constructed by ligating the Fen1 full-length cDNA (as a *Nco*I-*Sal*I fragment) into pET3a. pET-Fen1B was constructed by ligating the *Nco*I-*Bam*HI fragment of Fen1 cDNA into pET3a, resulting in the deletion of C-terminal 17 amino acids from full-length Fen1. All plasmids were transformed into *E. coli* BL21 for protein expression.

Bacterially expressed Fen1 was purified by chromatography on a DEAE-Sepharose column in buffer A (20 mM Hepes, pH 8.0/1 mM EDTA/0.1% Nonidet P-40/1 mM DTT/1 mM phenylmethylsulfonyl fluoride/10% glycerol) containing 0.3 M KCl (Flow-through) followed by binding to S-Sepharose at 0.1 M KCl and elution with 0.3 M KCl. This crude preparation of Fen1 protein was dialyzed against buffer A and loaded on 5-ml Mono-Q FPLC column (Pharmacia). Proteins were eluted with 50 ml of buffer A with a salt gradient of 0–1 M KCl. Fen1 peak was followed by SDS/PAGE and Coomassie staining.

For Fen1B purification, the flow through from the DEAE column was loaded on Q-Sepharose column (Pharmacia) equilibrated with buffer A containing 0.3 M KCl. The flow through from the Q-Sepharose was loaded on a 5-ml hydroxylapatite column (0.1 M KCl) and eluted with 15 ml of buffer A/0.1M KCl/80 mM sodium phosphate, pH 8.0. Eluted Fen1B was then dialyzed overnight against buffer A (pH 6.0) containing 25 mM KCl, loaded on a 5-ml S Sepharose column, and eluted with buffer A (pH 6.0) containing 100 mM KCl. Purified Fen1 or Fen1B were dialyzed against buffer A7.4 (20 mM Tris-HCl, pH 7.4/1 mM EDTA/0.01% Nonidet P-40/10% glycerol/25 mM NaCl/1 mM DTT/0.1 mM phenylmethylsulfonyl fluoride).

pETp21His expresses human p21 with a six-histidine tag at the N-terminal end. The purification of bacterially expressed Hisp21, Hisp27, and PCNA has been described (21, 30, 31).

The fragments containing full-length Fen1 and Fen1B were generated by PCR using *Pfu* polymerase (Stratagene) and cloned into pA3M vector (32) to generate pA3M-Fen1 and

pA3M-Fen1B for expression of myc epitope-tagged proteins in human cells.

Synthesis of Peptides. The synthetic peptide were purified using C₁₈ reverse-phase HPLC. p21C2 is the C-terminal 39 amino acids of p21 that binds PCNA (29). CSH190 is negative control peptide based on the sequence of Rpa2 (33).

Pull-Down Assay. Affinity chromatography on glutathione beads coated with various GST fusion proteins ("pull-down" assay) were done as described (34), except that washes were done with buffer A7.4. Bound proteins were eluted by boiling in Laemmli's buffer for 10 min, separated by SDS/polyacrylamide gel electrophoresis, and detected either by autoradiography (radiolabeled proteins) or by Western blot analysis using appropriate antibodies. In each experiment, care was taken to equalize the amount of input proteins, and GST protein was always included to serve as an negative control.

Gel Filtration and Glycerol Gradient. Proteins were incubated on ice for 15 min in A7.4 buffer before loading on a 25-ml Superose 12 gel filtration column (Pharmacia). Proteins were eluted from the column at flow rate 0.4 ml/min, and 0.5-ml fractions were collected.

Five-microliter 10–40% glycerol gradients were made with the gradient maker; 50–100 μ l of proteins was carefully added on the top of the gradient. Gradients were centrifuged at 55,000 rpm using SW55 rotor for 12–24 h in L8-M (Beckman). Fractions of 200 μ l were collected starting from the top of the gradients.

Exonuclease and Endonuclease Assay. Exonuclease and flap endonuclease assays were performed as described (9, 15).

Measurement of Binding Constants by BIAcore Instrument (Pharmacia). Detailed procedure of kinetic measurements by surface plasmon resonance technique using BIAcore instrument is described in manufacturer's instruction manual. The running buffer used for all binding experiments is 20 mM Hepes, pH 7.4/150 mM NaCl/3.4 mM EDTA/2 mM DTT/0.05% P-20 (Pharmacia). PCNA was immobilized on the surface of sensor chip using carbodiimide method by following manufacturer's instruction. For measuring binding parameters, five concentrations of Fen1 solutions were passed over the PCNA surface, and the affinity constant was estimated using software provided by the manufacturer.

Antibodies and Immunoprecipitations. Anti-Fen1 polyclonal rabbit antibody was raised against purified bacterial expressed Fen1 (Cocalico, Pennsylvania) and affinity-purified as described (35). PCNA was detected by Western blot analysis using a monoclonal antibody (PC10; Santa Cruz Biotechnology). The Myc-tagged Fen1 or Fen1B were transfected into 293T cells by the standard calcium phosphate coprecipitation method. Thirty-six hours after transfection, cells were lysed in Nonidet P-40 lysis buffer (22) and Myc-tagged proteins were immunoprecipitated using antibody (9E10) against the Myc epitope.

RESULTS

Human Fen1 Interacts with PCNA in a Two-Hybrid Assay.

A two-hybrid screen (28, 36) was carried out to search for human gene products that interacted with human PCNA. Yeast Y190 expressing a Gal4 DNA binding domain fused in-frame to human PCNA were transformed with a cDNA library from human B cells in the pACT vector. From 2×10^7 transformants, 60 positive clones were analyzed. Ten encoded p21 (four clones), and eight encoded Fen1 (two clones). The amount of β -galactosidase produced and the rate of growth on selective medium lacking histidine were equivalent for yeast expressing Fen1 or p21. The clones containing the human Fen1/Rad2 gene (11) expressed amino acids 249–380 (C terminus) or amino acids 115–380 of Fen1 (Fig. 1), suggesting that the C-terminal portion of Fen1 could be involved in the

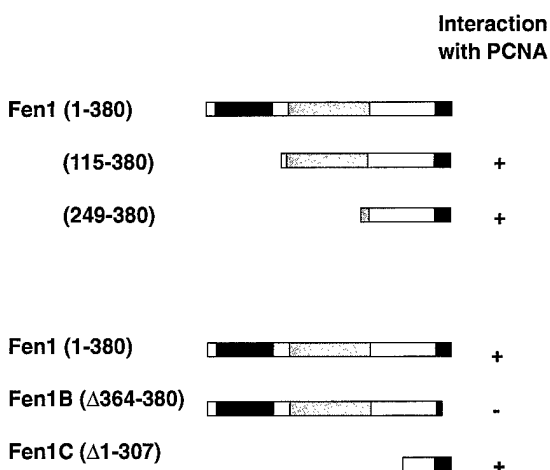


FIG. 1. Summary of two-hybrid screen and of Fen1 deletions used in this paper. (Upper) Two cDNAs (eight clones) encoded amino acids 115–380 and amino acids 249–380 of human Fen1 were isolated by two-hybrid screen using human PCNA as the bait. None of the Fen1 cDNA isolated interacts with any other baits tested in the two-hybrid assay. (Lower) Deletion derivatives used in this paper. The domains conserved between Fen1 and other exonucleases including XP-G are indicated by shaded and solid boxes in the Fen1 schematic.

interaction. None of the clones interacted with a negative control bait comprising the Gal4 DNA binding domain alone.

Fen1 Binds PCNA Through Its C-Terminal Tail. To verify the direct interaction between PCNA and Fen1, both proteins were expressed in *E. coli*. When PCNA was expressed as a GST fusion protein, Fen1 formed a stable complex with PCNA in the pull-down assay (Fig. 2A). But Fen1B, which lacks the basic C-terminal 17 amino acids of Fen1, fails to associate with PCNA (Fig. 2A). GST–Fen1C, which contains the C-terminal 77 amino acids, binds to PCNA (Fig. 2B, also see Fig. 5A). These results suggest that Fen1 associates with PCNA through its C-terminal tail.

Three Molecules of Fen1 Form a Complex with the PCNA Trimer. To ensure that the interaction occurred between Fen1 and a functional PCNA trimer, Fen1 and PCNA were produced and purified from a bacterial expression system (Fig. 3). Proteins used in the experiments were pure of any contaminant proteins (data not shown). PCNA was eluted from a Superose 12 gel filtration column as a complex of approximately 150 kDa (Fig. 3A). Fen1 eluted as a 45-kDa protein. When PCNA and

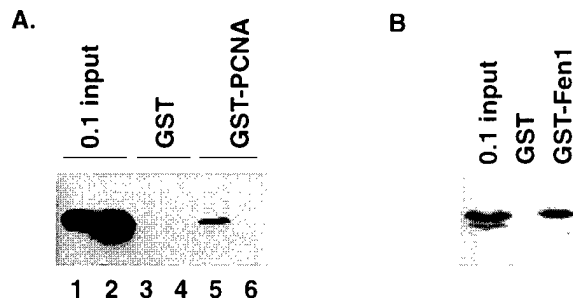
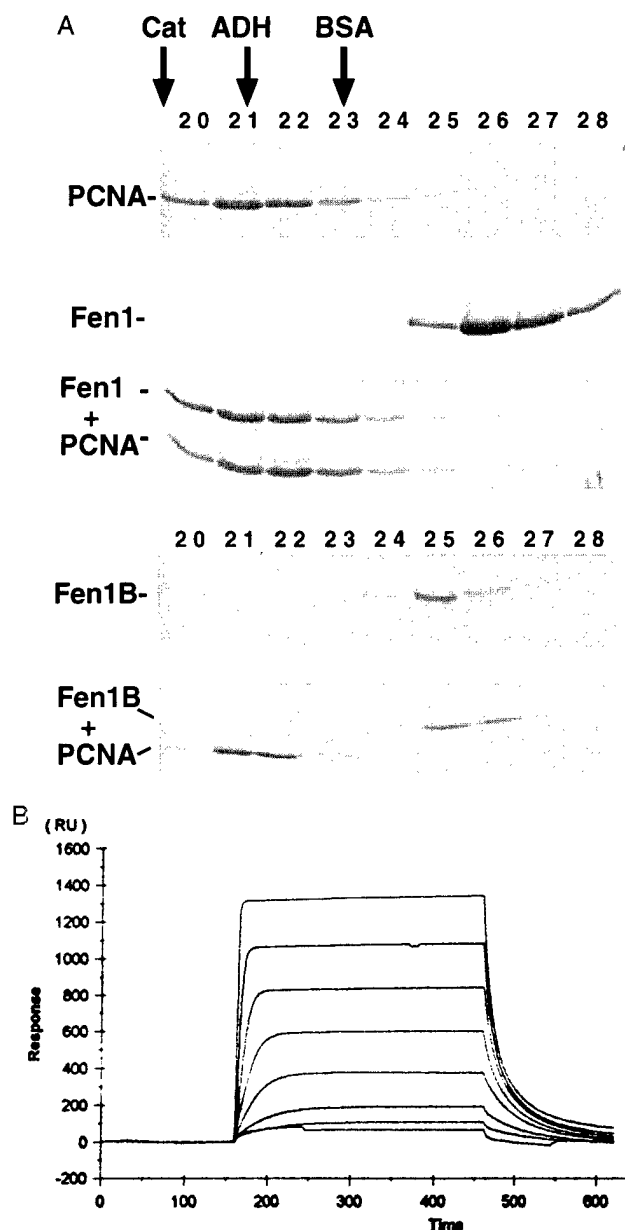


FIG. 2. PCNA interacts with the C-terminal tail of Fen1. (A) Bacterial lysates containing either Fen1 (lanes 1, 3, and 5) or Fen1B (lanes 2, 4, and 6) were incubated with glutathione-agarose beads containing GST (lanes 3 and 4) or GST–PCNA (lanes 5 and 6). Lanes 1 and 2 were, respectively, the 10% of Fen1 or Fen1B lysates used in the experiment. Bound proteins were visualized by Western blot analysis using polyclonal rabbit antibody against Fen1. (B) PCNA was labeled with [³⁵S]Met using a coupled *in vitro* transcription and translation system (Pharmacia). Lysate containing PCNA was incubated with either GST or GST–Fen1C beads. Bound PCNA was visualized by fluorography.



The interaction occurs through the basic tail of Fen1 and does not disrupt the trimeric structure of PCNA that is essential for its function as a processivity factor for DNA polymerase δ .

The stoichiometry of the Fen1–PCNA interaction was determined by mixing Fen1 with PCNA at various molar ratios and determining by sedimentation on a glycerol gradient whether free Fen1 (sedimenting at 45 kDa) was present in the mixture. Free Fen1 was detected only when the ratio of Fen1 to PCNA exceeded three molecules of Fen1 per PCNA trimer (data not shown). Therefore, three molecules of Fen1 bind to each PCNA trimer.

Native molecular mass of PCNA trimer and Fen1–PCNA complex were calculated based on the results of the gel filtration experiments and glycerol gradient sedimentation experiments. The calculated molecular mass of Fen1–PCNA complex is 209 kDa, while that of PCNA trimer is 123 kDa (although the theoretical molecular weight of PCNA trimer is 88 kDa). This would suggest two Fen1 molecules associate with one PCNA trimer. Because of the abnormal behavior of PCNA in gel filtration experiments and the results presented above and following, we favor that three molecules of Fen1 associate with one PCNA trimer.

The affinity of the Fen1–PCNA interaction was determined by surface plasmon resonance technique using BIAcore instrument. The 2627 resonance units (RU) of PCNA trimer were immobilized on the sensor chip and solutions containing Fen1 at various concentrations was passed over PCNA-coated chip in the BIAcore machine. The sensorgrams (Fig. 3B) indicated that the K_d of the interaction was about 60 nM at 25°C. The stoichiometry of the interaction (based on the maximum number of resonance units of Fen1 that bind to a fixed number of resonance units of PCNA) was about 2.47 molecules of Fen1 per PCNA trimer. Since all the PCNA immobilized on the chip may not remain functionally active, this ratio is consistent with the stoichiometry determined by glycerol-gradient sedimentation (three molecules of Fen1 per PCNA trimer).

PCNA Interacts with Fen1 *in Vivo* and the Interaction Is Mediated by the C Terminus of Fen1. Fen1 was immunoprecipitated from cell extracts and the precipitate was probed with anti-PCNA antibody to reveal that PCNA was coprecipitated (Fig. 4A). This suggests that the two proteins associate with each other *in vivo* under physiologically relevant conditions. If the interaction between Fen1 and PCNA *in vivo* was direct, then the C terminus of Fen1, which was required for direct interaction *in vitro*, would be equally crucial *in vivo*. Wild-type and deletion derivatives of Fen1 with a myc epitope tagged to the N terminus were expressed in cells by transient transfection and the presence of PCNA in anti-myc immunoprecipitates was tested (Fig. 4B). Deletion of the C terminus of Fen1 (Fen1B; Fig. 1) disrupted association with PCNA *in vivo*.

PCNA Does Not Stimulate the Exonuclease and Endonuclease Activities of Fen1. As PCNA forms a stable complex with Fen1, we tested whether PCNA can regulate Fen1's activity. On a poly(dA)·oligo(dT) substrate, addition of PCNA inhibited the exonuclease activity of Fen1 at a 100- to 500-fold excess of PCNA. Because this result contradicts the reported 10-fold stimulation of yeast Fen1 by yeast PCNA (37), we examined other DNA substrates for Fen1. On the hairpin template, PCNA increased the exonuclease activity of Fen1 by 2-fold at high concentration (100- to 500-fold of excess of PCNA). We do not think this modest stimulation is significant. (i) The molar ratio of PCNA trimer and Fen1 in these reactions was about 100–500 to 1, in contrast to the stoichiometry of PCNA trimer–Fen1 complex, which was 1 to 3. (ii) A 2-fold stimulation by high concentration of PCNA was also observed when Fen1B was used in the reactions, suggesting the stimulation is not due to the association between Fen1 and PCNA. (iii) When purified Fen1–PCNA complexes were used, the complexes had similar activity as Fen1 (data not shown).

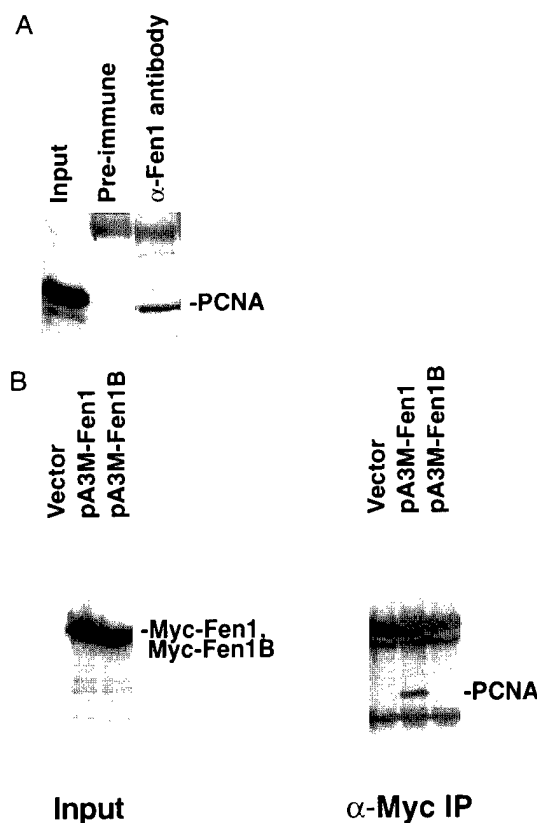


FIG. 4. Fen1–PCNA interactions *in vivo*. (A) One milligram of H1299 cell lysates was immunoprecipitated with indicated antibodies and the precipitates were examined by immunoblot analysis with anti-PCNA antibody. (B) Cell lysates containing the indicated proteins (Left) were immunoprecipitated with anti-myc antibody 9E10 (Right) and precipitates were examined by immunoblot analysis with anti-PCNA antibody. The C-terminal portion of Fen1 (absent in Fen1B) was required for association with PCNA *in vivo*.

PCNA did not stimulate the Flap endonuclease activity of Fen1 either (data not shown). Based on these results, we do not think that human PCNA stimulates the enzymatic activities of Fen1.

p21 Displaces Fen1 from the Fen1–PCNA Complex. Since p21 also interacts with PCNA using a basic region of p21 (38, 39) similar to that used by Fen1, we tested whether the two molecules compete for the same binding site on PCNA. p21 displaced PCNA from the GST–Fen1C molecule (Fig. 5A). Further, p21C2 (29), a synthetic peptide that contains the C-terminal 39 amino acids of p21 and that interacts with and inhibits PCNA, also disrupted the Fen1C–PCNA complex.

The mutually exclusive complex formation between PCNA–Fen1 and PCNA–p21 were further demonstrated by gel filtration assay (Fig. 5B). GST–p21C2 (31 kDa) is a fusion of the last 39 amino acids of p21 with GST and associates well with PCNA (29, 39). When GST–p21C2, Fen1 (45 kDa), and PCNA (120 kDa) were mixed and analyzed on a gel filtration column, all three proteins eluted in the same fractions of about 150 kDa (Fig. 5B). However, when the GST–p21C2 was precipitated from these fractions with glutathione-agarose beads, only PCNA was coprecipitated (Fig. 5C), suggesting that the three proteins were present in two separate complexes, each of about 150 kDa, GST–p21C2–PCNA and Fen1–PCNA. A tripartite Fen1–p21–PCNA complex was never observed.

To demonstrate that p21 can disrupt Fen1–PCNA interaction *in vivo*, Myc-tagged Fen1 was expressed alone or with p21 in the cells. While Myc-tagged Fen1 associated with PCNA *in vivo*, coexpression of p21 in these cells disrupted Fen1–PCNA interaction (Fig. 5D).

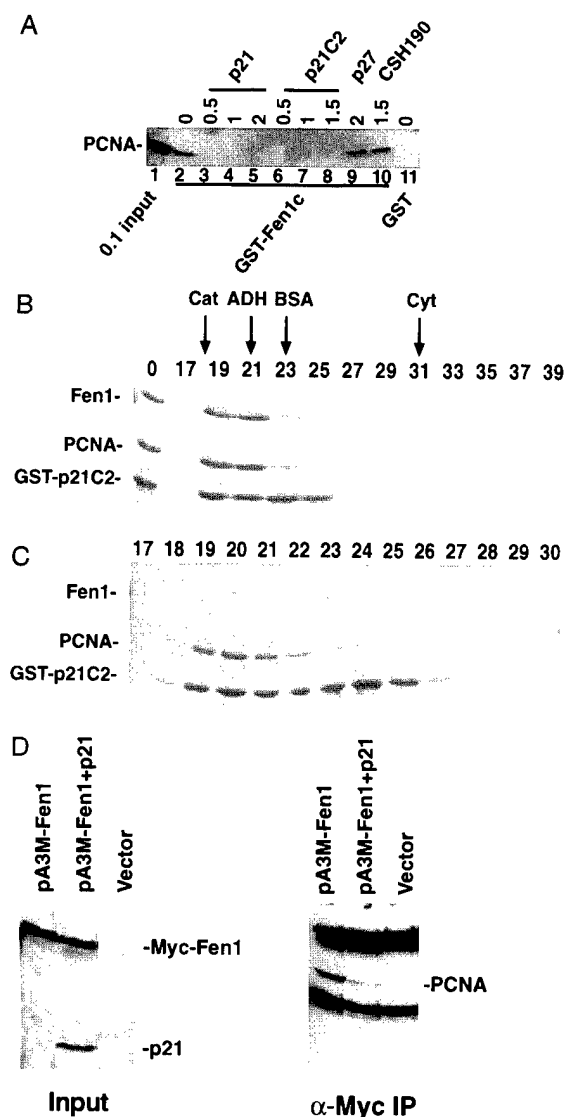


FIG. 5. p21 disrupts Fen1-PCNA interaction. (A) Bacterial lysate containing human PCNA was incubated with the GST-Fen1C or with GST immobilized on the agarose beads in a pull-down assay. PCNA bound to the beads were detected by immunoblot analysis with anti-PCNA antibody. Lane 1, 10% input lysate; lanes 2-10, GST-Fen1C beads incubated with bacterial lysate containing PCNA with no additional protein (lane 2), with additional Hisp21 (lanes 3-5; 0.5, 1, and 2 μ g), p21C2 peptide (lanes 6-8; 0.5, 1, and 2 μ g), Hisp27 (lane 9; 2 μ g), or control peptide CSH190 (lane 10; 1.5 μ g); lane 11, GST beads incubated with PCNA containing lysate. (B) A mixture of 25 μ g of PCNA (36 kDa), 25 μ g of Fen1 (45 kDa), and 50 μ g of GST-p21C2 (31 kDa) was analyzed on a Superose 12 gel-filtration column. (C) The indicated fractions (200 μ l) from B were incubated with glutathione-agarose beads, and bound proteins (GST-p21C2 and associated proteins) were separated by SDS/PAGE and visualized by Coomassie staining. (D) Cells were transfected with Myc-Fen1 alone, Myc-Fen1 and p21, or vector alone and collected after 36 h. Cell lysates were examined by immunoblot analysis with anti-Myc and anti-p21 antibodies to demonstrate that equal levels of Myc-Fen1 are expressed in the first two lanes (Left). Lysates were immunoprecipitated with anti-myc antibody 9E10 and precipitates were examined by immunoblot analysis with anti-PCNA antibody to demonstrate that coexpression of p21 decreases Myc-Fen1-PCNA association (Right).

DISCUSSION

The crucial role played by Fen1 in DNA replication has become evident over the last year. However, it has been unclear as to how the enzyme enters the replication machinery. Our results emphasize the central role of PCNA as the scaffold

that recruits and retains various replication proteins to the machinery at the replication fork. The stable association of Fen1 with PCNA, which is itself associated with polymerase δ , confers on this complex all three activities noted in prokaryotic DNA replication polymerases: 5'-3' DNA polymerase, 3'-5' exonuclease (both from polymerase δ), and 5'-3' exonuclease (from Fen1). Waga and Stillman (5) have reported that even with the addition of Fen1 to an simian virus 40 (SV40)-based DNA replication reaction, covalently closed product was not obtained unless PCNA, RF-C, and polymerase δ were also added. Covalently closed products were also not obtained when a prokaryotic leading-strand synthesis enzymes were added with Fen1 (5). The direct physical interaction between PCNA and Fen1 reported herein probably explains why PCNA must be present to target Fen1 to the growing end of an Okazaki fragment. In addition, the stable association of yeast Fen1 with a putative DNA replication helicase (40) implies that the Fen1-PCNA interaction may also be indirectly responsible for tethering a replication helicase to the replication fork.

The interaction between Fen1 and PCNA was also discovered in yeast (37). Yeast PCNA can stimulate the exonuclease and Flap endonuclease activity of yeast Fen1, but it is not the case with human counterparts. This may reflect the differences between different species. We note that the reported 10-fold stimulation of yeast Fen1 activity was observed using 500-fold molar excess of yeast PCNA trimer. However, in our experiments, the 2-fold stimulation of Fen1 by 100-fold excess of PCNA was also seen with Fen1B, which does not associate with PCNA. Hence, we believe that human Fen1 is not stimulated by human PCNA.

A novel function of p21 on the DNA replication apparatus has emerged from these studies. By interacting with PCNA, p21 affects the processivity of the polymerase δ complex (20, 25, 41). Since this activity is noted with pure PCNA, polymerase δ , and p21, in the absence of Fen1, we believe that the displacement of Fen1 is an additional activity of p21, independent of the stalling of polymerase δ . The ability of p21 to disrupt Fen1-PCNA complex is consistent with the K_d of p21-PCNA interaction (15 nM; ref. 29) being less than that of Fen1-PCNA (60 nM, this paper).

Glycerol-gradient sedimentation and surface plasmon resonance spectroscopy shows that three molecules of Fen1 associate with a PCNA trimer. Three molecules of p21 associate with a PCNA trimer (20, 29). Therefore, if the displacement of Fen1 from PCNA by p21 was merely due to the occupation of the same site on PCNA, one expects to see intermediate complexes where p21 and Fen1 were both associated with the same PCNA trimer. The fact that this does not occur strongly suggests that Fen1 or p21 induce changes in the PCNA structure such that PCNA trimer can only associate with either Fen1 or p21 at all three sites.

By selectively inhibiting the synthesis of long DNA strands by PCNA-polymerase δ (as required for replication) while permitting the synthesis of short DNA strands (as required for repair synthesis), p21 plays an important role of favoring repair over replication (20, 25, 26, 41). The removal of Fen1 from the PCNA-polymerase δ complex will also shift the balance in favor of repair vs. replication because the short patches of DNA synthesized during repair can be ligated to DNA 3' to the excised lesion without requiring the removal of RNA primers and, hence, without requiring Fen1.

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